THE REGULATION OF CELL-MATRIX ADHESION IN MOUSE FIBROBLASTS

by

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Abbreviations

Amp  ampicillin
ATP  adenosine triphosphate
BSA  bovine serum albumin
bp   base pairs
°C   degrees centigrade
cAMP cyclic adenosine monophosphate
cDNA complimentary DNA
CEF  chick embryo fibroblasts
cGMP cyclic guanosine monophosphate
CHO  chinese hamster ovary cells
Ci   curie
CNBr cyanogen bromide
CO₂  carbon dioxide
cet  cultured cells
DMSO dimethyl sulphoxide
DMEM dulbecco's modified eagle medium
DNA deoxyribonucleic acid
dNTP deoxyribonucleotide triphosphate
DTT  dithiothreitol
E. Coli Escherichia coli
ECM extracellular matrix
EDTA diaminoethane-tetra-acetic acid
EGTA ethyleneglycol-bis-(β-aminoethyl ether)N, N, N', N'-tetra-acetic acid
ERM  ezrin, radixin, moesin
FCS  foetal calf serum
FITC fluorescein isothiocyanate
FRNK focal adhesion kinase related non-kinase
GAP  GTPase activating protein
GTP  guanosine triphosphate
HCl  hydrogen chloride
HEPES 2-[N-morpholinol]-ethanesulfonic acid
HRP  horse radish peroxidase
IPA  isopropyl alcohol
JNK  jun N-terminal kinase
LC20 myosin light chain
LPA lysophosphatidic acid
kb   kilobase
<table>
<thead>
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<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>SV40</td>
<td>simian virus 40</td>
</tr>
<tr>
<td>TBS</td>
<td>tris buffered saline</td>
</tr>
<tr>
<td>TEMED</td>
<td>N, N', N', N'-tetramethylethylenediamine</td>
</tr>
<tr>
<td>TPA</td>
<td>tetradecanoylphorbol 13-acetate</td>
</tr>
<tr>
<td>Tris</td>
<td>(hydroxymethyl)aminomethane</td>
</tr>
<tr>
<td>TRITC</td>
<td>Texas Red™ isothiocyanate</td>
</tr>
<tr>
<td>Tween 20</td>
<td>polyoxyethylene sorbitan monolaurate</td>
</tr>
</tbody>
</table>
Abstract

Regulation of cell-matrix adhesion in mouse fibroblasts
Simon Thomas Barry

Mouse Swiss 3T3 fibroblasts maintained in serum-free medium lose their actin stress fibres and focal adhesions. Addition of serum or lysophosphatidic acid (LPA) to these cells induced the rapid reformation of these structures, and the increased tyrosine phosphorylation of pp125Fak and paxillin. The formation of focal adhesions and actin stress fibres was shown to require tyrosine phosphorylation; it was blocked by the tyrosine kinase inhibitor genistein, and stimulated by the tyrosine phosphatase inhibitors sodium orthovanadate and phenyl arsine oxide. Moreover, the activity of a tyrosine phosphatase directed against pp125Fak and paxillin was elevated in serum-starved cells.

Despite the absence of focal adhesions and actin stress fibres, the adhesion of serum-starved cells was shown to be mediated by integrins, as peptides corresponding to the cell-binding motif of fibronectin caused cell detachment from the extracellular matrix. In addition, the integrin-mediated formation of actin stress fibres and protein tyrosine phosphorylation was blocked by introducing C3 transferase into cells, a result which establishes a role for the small GTP-binding protein rho in signalling via integrins.

PKC was also required to maintain adhesion in serum-starved cells as the PKC inhibitor calphostin C caused cell retraction, a process that was accompanied by the formation of actin stress fibres. In contrast, activation of PKC with phorbol esters suppressed the LPA-induced assembly of actin stress fibres and focal adhesions, and caused loss of these structures in cells grown in serum. The results suggest that PKC negatively regulates the formation of actin stress fibres.

The proposed role of vinculin phosphorylation in the formation of focal adhesions was found to be inconsistent with the finding that the phosphorylated form of the protein is not associated with the Triton X-100 insoluble fraction in Swiss 3T3 cells. Vinculin was a poor substrate for PKC in vitro, although a cryptic C-terminal phosphorylation site was exposed by PIP2.
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Chapter One

Introduction
1.1 General introduction

1.1.1 Background

Cell adhesion to the extracellular matrix is fundamental to a wide variety of biological phenomena. It is required for the migration and differentiation of cells to form organs and tissues during embryogenesis (McClay and Ettensohn, 1987), is important for the wounding response, in particular in the formation of a blood clot by activated platelets (Shattil et al., 1994), and is fundamental to the immune response when T-cells and B-cells are activated (Springer, 1990), or where leukocytes migrate to a site of inflammation (Stewart et al., 1995). Loss of the requirement for adhesion is synonymous with diseased states. Absence of the β2-integrin subunit alters the adhesion between leukocytes and endothelial cells rendering the leukocyte incapable of migrating to sites of inflammation (Huttenlocher et al., 1995). Disrupted adhesion is also common in the development of a variety of pathological diseases including inflammatory diseases, atherosclerosis and metastatic tumour development. The most widely discussed of these is probably the development of metastatic tumours. Cells such as transformed epithelial cells, that have lost the requirement of adhesion for growth, can break off from a solid tumour and migrate to other sites, via nearby blood vessels and the lymphatic system, where they can invade tissues or organs, adhere and proliferate in an unregulated manner, forming a secondary tumour (Liotta et al., 1986).

Adhesion and migration of cultured cells on the underlying substratum was initially studied in fibroblasts isolated from chick heart explants (Abercrombie et al., 1970a; Abercrombie et al., 1970b). Fibroblasts were highly motile when plated on glass coverslips, moving by extending a thin sheet of cytoplasm known as the leading edge or lamella. These studies also revealed that the ventral surface of the cells did not lie flat to the substrate but rather appeared to interact via numerous electron dense areas which brought the cytoplasm to within 30 nm of the coverslip and into which microfilament bundles appeared to insert (Abercrombie et al., 1971). Such regions were also subsequently identified in non-motile cells by interference reflection microscopy (Abercrombie and Dunn, 1975). These regions of close contact are the focal adhesions visualised by immunofluorescence, and the microfilament system that inserts into them, the associated actin stress fibre. The importance of these regions for cell motility was highlighted more recently with the demonstration that cytoskeletal proteins are preferentially recruited to adhesion points at the leading edge (lamella), and as the cell pulls itself forward these adhesions appear to move through to the rear of the cell (Schmidt et al., 1993).

Early work on cultured primary chick fibroblasts established the importance of the adhesion as a signalling complex. Transition from a motile cell, containing few smaller adhesions, to a stationary cell with more larger adhesions was associated with entry into G1 of the cell cycle (Couchman et al., 1982). Increasing the concentration of substrate
fibronectin decreased motility and increased proliferation. Indeed increased cellular
fibronectin expression also induced reduced migration and entry into the cell cycle.
Moreover disrupting adhesion of Balb/c 3T3 cells by taking them into suspension induced
entry into G0 of the cell cycle. Subsequently plating these cells on fibronectin induced the
expression of growth-associated genes (Dike and Farmer, 1988). Adhesion is significantly
reduced when cells are virally transformed with the v-src oncogene (Tarone et al., 1985).
The importance of perturbation of the normal adhesive machinery in cellular
transformation has been recently demonstrated. Overexpression of α5β1 integrin in colon
carcinoma cells decreases proliferation and loss of the transformed phenotype when the
cells were plated on fibronectin (Varner et al., 1995). Understanding the mechanism
through which the cell interacts with the ECM is fundamental to understanding a number
of disease states.

1.1.2 The focal adhesion
Cells in culture often have a requirement for adhesion to a solid support to facilitate normal
growth. Adherent cell lines adapted for growth in culture can interact with a variety of
extracellular matrix (ECM) proteins, such as fibronectin or vitronectin, through regions of
close adhesive contacts on the ventral surface of the cell. This focal adhesion (also known
as the cell-matrix junction or adhesion plaque) is commonly regarded as an artifact, a result
of growing cells in culture. However structures analogous to the focal adhesion occur in a
number of cell types in vivo. Upon platelet activation and aggregation, the majority of the
components of the focal adhesion are recruited to the adhesive structures that interact to
form the blood clot (Lipfert et al., 1992; Vostal and Shulman, 1993; Bertagnolli et al.,
1993a; Shattil et al., 1994; Clark and Brugge, 1995). The myotendenous junctions of
skeletal muscle also share common proteins and properties with focal adhesions (Shear and
Bloch, 1985; Tidball et al., 1986; Turner et al., 1991; Bockholt et al., 1992; Baker et al.,
1994), as do the dense plaques found in smooth muscle (Small, 1985).

For a number of years the structure of the focal adhesion was regarded as a rather simple
macromolecular complex through which ECM proteins such as fibronectin or vitronectin
were ultimately linked to the actin cytoskeleton. Cells adhere to ECM components via a
family of transmembrane receptors known as integrins, heterodimeric glycoproteins that
consist of a variety of α and β subunits which act in concert to mediate specific cellular
interactions (Hynes, 1992). On the cytoplasmic face of the membrane the integrins interact
with a complex macromolecular array of proteins. Under the cytoplasmic face of the
ventral membrane the cytoplasmic tail of the β1-integrin is capable of interacting with both
the 210 kDa actin binding protein talin (Horwitz et al., 1986; Muguruma et al., 1990) and
110 kDa actin binding and crosslinking protein α-actinin (Pavalko et al., 1991). Both
proteins are in turn able to interact with the 116 kDa actin binding protein vinculin
(Burridge and Mangeat, 1984; McGregor et al., 1994). These four proteins were originally
modelled to form the structural link anchoring the actin cytoskeleton to the membrane, by virtue of their actin binding properties. Over recent years the number of new proteins known to localise to the focal adhesion has rapidly expanded, including a number of signalling proteins such as the tyrosine kinases focal adhesion kinase (pp125Fak) and src family members, and the protein kinase C (PKC) family of serine/threonine kinases. These signalling proteins not only control the assembly of adhesive structures but also exert effects on signalling pathways controlling both cell division and differentiation. Cells in culture are therefore at present considered the simplest and most amenable system with which to study many aspects of cell adhesion. The structural and signalling proteins associated with mediating cell matrix adhesion are show in Table 1.1. A simplistic model of the focal adhesion showing the major proteins and their various interactions are represented in Fig. 1.1. Importantly the adhesion of a number cells to the ECM is elicits a variety of intracellular signalling events. Integrin engagement results in a rise in intracellular pH and calcium, and inositol lipid production (Juliano and Haskill, 1993; Schwartz et al., 1995) and gene expression (Werb et al., 1989). Engagement of the ECM by fibroblasts stimulates the the expression of mRNA encoding the proteases collagenase and stromelysin (Werb et al., 1989; Huhtala et al., 1995).

**Components of cell-matrix junctions**

1.2 Extracellular matrix and integrins

1.2.1 The extracellular matrix

Adhesion receptors interact with a diverse set of structural proteins that make up the extracellular matrix. These molecules control cell behaviour, migration, proliferation, differentiation and ultimately the organisation of different cell types to form tissues and organs. For example the structure and integrity of tissues and organs is dependent on collagen fibrils. This family of ECM proteins, along with other proteoglycans and glycoproteins, form a three dimensional scaffold, supporting and organising cells into structures. The nature of this 3-D scaffold is in part determined by the collagen isoform present in the matrix (reviewed in Olsen, 1994). The ECM proteins are large adhesive glycoproteins, and many are secreted by fibroblasts. ECM proteins include fibronectin, laminin, vitronectin, fibrinogen (platelets), von Willebrand factor, entactin, thrombospondin and collagens (reviewed Yamada, 1991). Cells interact with these large proteins via specific short peptide motifs. Such motifs form specific ligands for individual integrin heterodimers, with a number of different integrin heterodimers capable of interacting with different regions of various ECM molecules.
Table 1.1 Proteins involved in the structure and regulation of cell adhesion.

<table>
<thead>
<tr>
<th>Structural Proteins</th>
<th>Molecular Weight kDa</th>
<th>Interactions and Functions</th>
</tr>
</thead>
<tbody>
<tr>
<td>Integrins</td>
<td></td>
<td>See Table 1.2. The extracellular domain of integrin bind ECM components such as fibronectin and vitronectin. The cytoplasmic tail of β1-integrin binds α-actinin, talin, pp125Fak and paxillin. Links cytoskeleton with the extracellular matrix.</td>
</tr>
<tr>
<td>Talin</td>
<td>210</td>
<td>Binds β1 integrin, vinculin, pp125Fak, paxillin and actin. Acts as an F-actin nucleating protein. Member of the band 4.1 family. Possible interaction with membrane.</td>
</tr>
<tr>
<td>Vinculin</td>
<td>116</td>
<td>Binds talin, α-actinin, paxillin and actin. Possible interaction with acidic phospholipids. Head binds talin to compromise other interactions.</td>
</tr>
<tr>
<td>Tensin</td>
<td>205</td>
<td>Binds vinculin, and actin. Tyrosine phosphorylated and contains SH2 domain. Actin barbed end capping protein, allow slow growth of actin filaments.</td>
</tr>
<tr>
<td>VASP</td>
<td>46/50</td>
<td>Binds profilin and actin. Thought to regulate the addition of actin monomers to growing filaments.</td>
</tr>
<tr>
<td>Radixin</td>
<td>82</td>
<td>Binds actin and possibly the plasma membrane. N-terminal region homologous to talin. Actin barbed end capping protein. Member of the band 4.1 family.</td>
</tr>
<tr>
<td>Ezrin</td>
<td>85</td>
<td>Binds actin and possibly the plasma membrane. Shows homology to talin. Actin barbed end capping protein. Member of the band 4.1 family.</td>
</tr>
<tr>
<td>Moesin</td>
<td>78</td>
<td>Binds actin. Member of the band 4.1 family.</td>
</tr>
<tr>
<td>Dystrophin</td>
<td>427</td>
<td>Binds F-actin, calcium and the plasma membrane. Links the sides of actin to the plasma membrane via a glycoprotein complex. Homologous to utrophin.</td>
</tr>
<tr>
<td>Utrophin</td>
<td>395</td>
<td>Binds F-actin, calcium and the plasma membrane. Links the sides of actin to the plasma membrane via a glycoprotein complex. Homologous to dystrophin.</td>
</tr>
<tr>
<td>Actin</td>
<td>43</td>
<td>Exists as filamentous F-actin or monomeric G-actin. F-actin interacts with numerous cytoskeletal proteins. G-actin can be sequestered by profilin and MARCKS.</td>
</tr>
<tr>
<td>pp110AFAP</td>
<td>110</td>
<td>Actin filament associated protein. Tyrosine phosphorylated protein. Interacts with src and fyn via the SH2/SH3 domains.</td>
</tr>
<tr>
<td>pp130CAS</td>
<td>130</td>
<td>Tyrosine phosphorylated protein. Interacts with the SH2 domains of v-er and v-src. Contains a SH3 domain.</td>
</tr>
<tr>
<td>Profilin</td>
<td>12-15</td>
<td>Actin and PIP2 sequestering protein. Plays a role in regulates the availability of actin monomers for actin polymerisation.</td>
</tr>
<tr>
<td>Enzymatic Proteins</td>
<td>Molecular Weight (kDa)</td>
<td>Interactions and Functions</td>
</tr>
<tr>
<td>------------------------------------</td>
<td>------------------------</td>
<td>----------------------------------------------------------------------------------------------------------------------------------------------------------</td>
</tr>
<tr>
<td>pp60 src</td>
<td>60</td>
<td>Tyrosine kinase. Binds paxillin, pp125Fak, pp110AFAP and possibly pp130CAS. Phosphorylates numerous cytoskeletal proteins.</td>
</tr>
<tr>
<td>Src family members fyn lek yes etc.</td>
<td>53-59</td>
<td>Family of approx 9 tyrosine kinases related to src. Fyn also binds paxillin and pp125Fak.</td>
</tr>
<tr>
<td>pp125Fak</td>
<td>125</td>
<td>Novel tyrosine kinase. Binds β1-integrin, p85 subunit of PIP3 kinase, paxillin, talin, grb2, fyn, src and csk. Thought to regulate aspects of adhesion formation.</td>
</tr>
<tr>
<td>Protein kinase C</td>
<td>75-80</td>
<td>Family of 9 or more serine threonine kinases. Possibly interacts with vinculin and α-actinin. Required for cells to spread on fibronectin and for the formation of focal adhesions and stress fibres.</td>
</tr>
<tr>
<td>Calpain II</td>
<td>80</td>
<td>Calcium dependent protease. No known binding partners with the focal adhesion. Cleaves talin, c-src PTP1B in platelets and PKC isoforms.</td>
</tr>
<tr>
<td>Heterotrimeric G protein γ subunit</td>
<td>7</td>
<td>Couples the heterotrimeric G-protein transmembrane receptor to its effector. Isoprenylated for membrane localisation.</td>
</tr>
<tr>
<td>LAR phosphatase</td>
<td>200</td>
<td>Transmembrane tyrosine phosphatase. Function and substrates unknown. Consists of two subunits 150 and 85 kDa.</td>
</tr>
<tr>
<td>Csk</td>
<td>50</td>
<td>Tyrosine kinase, negatively regulates src. Binds pp125Fak and paxillin. Contains SH2 and SH3 domains. Localised to focal adhesions when overexpressed in cells.</td>
</tr>
<tr>
<td>PLCγ</td>
<td>145</td>
<td>Recruited to complexes formed upon binding of FN coated beads to cells. Hydrolyses PIP2 to generate DAG and IP3, activating PKC and releasing intracellular calcium. Regulated by rhoA.</td>
</tr>
<tr>
<td>PI 3-kinase</td>
<td>85 and 110</td>
<td>85 kDa subunit recruited to complexes formed upon binding of FN coated beads to cells. Phosphorylates inositol lipids at position D3. p85 subunit of this enzyme contains SH2 and SH3 domains. Binds pp125Fak, src and α-actinin. Activity is regulated in vitro by rho.</td>
</tr>
<tr>
<td>Rho</td>
<td>21</td>
<td>Small molecular weight GTPase. Localises to plasma membrane through isoprenylation. Controls the actin cytoskeleton and adhesion formation. No binding partners known.</td>
</tr>
<tr>
<td>Rac</td>
<td>21</td>
<td>Small molecular weight GTPase. Localised by inference. Controls membrane ruffling. No known binding partners within the adhesion.</td>
</tr>
<tr>
<td>Ras</td>
<td>21</td>
<td>Small molecular weight GTPase. Localised by inference. Associated with the plasma membrane by isoprenylation. No binding partners demonstrated within the adhesion.</td>
</tr>
<tr>
<td>PIP 5-kinase</td>
<td></td>
<td>Phosphorylates PIP to yield PIP2, and PIP2 to yield PIP3. Activity regulated by rho in cell free assays. Has not been localised to adhesive complexes.</td>
</tr>
<tr>
<td>Phospholipase D</td>
<td></td>
<td>Catalyses the hydrolysis of phosphatidylcholine to phosphatidic acid and choline. Regulated by rho in vitro. Has not been localised to adhesive complexes.</td>
</tr>
<tr>
<td>Other proteins</td>
<td>Molecular Weight kDa</td>
<td>Interactions and Functions</td>
</tr>
<tr>
<td>---------------</td>
<td>----------------------</td>
<td>---------------------------</td>
</tr>
<tr>
<td>Zyxin</td>
<td>82</td>
<td>Binds α-actinin, VASP, cCRP. Contains LIM domains. No known function.</td>
</tr>
<tr>
<td>cCRP</td>
<td>23</td>
<td>Binds cCRP. Contains LIM domains. No known function.</td>
</tr>
<tr>
<td>FRNK</td>
<td>41</td>
<td>Fak related non kinase. Autonomously expressed, alternatively spliced portion of ppl25Fak corresponding to the C-terminal region.</td>
</tr>
<tr>
<td>Crk</td>
<td>47</td>
<td>Adaptor protein, contains SH2 and SH3 domains. Binds paxillin and the ras exchange factor C3G. Thought to couple adhesions to small GTP-binding proteins.</td>
</tr>
</tbody>
</table>
Figure 1.1. Schematic representation of the focal adhesion. The possible interactions of the major structural and signalling proteins known to localise and interact at the focal adhesion are represented. Four possible links are shown. A- β1 integrin, vinculin and actin. B- β1-integrin, α-actinin and actin or β1-integrin, α-actinin, vinculin and actin. C- β1-integrin, talin and actin. pp125Fak is represented binding to both the cytoplasmic tail of β1-integrin and talin, while paxillin and zyxin bind vinculin and α-actinin respectively.
1.2.2 Fibronectin

Fibronectin is a ubiquitous extracellular glycoprotein found in a soluble form in body fluid and an insoluble form in the ECM. It is secreted as a dimer, each monomer consisting of approximately 220-250 kDa, which are joined at the C-terminus by a pair of disulphide bridges (Potts and Campbell, 1994). The protein consists of a number of modules (Engel, 1991), represented in Fig. 1.2. These domains contain binding sites for extracellular matrix protein such as collagen and thrombospondin, cell-surface receptors (e.g. integrins) and a variety of circulating blood proteins such as fibrin and glycosaminoglycans such as heparin and chondroitin sulphate (Potts and Campbell, 1994). These protein-protein interactions enable fibronectin to form part of the mesh-like clot assembled during wound healing.

Fibronectin contains two cell-binding domains, the first identified being the classical central cell-binding domain. This comprises a number of type 3 repeats harbouring an Arg-Gly-Asp-Ser (RGDS) motif within the tenth repeat. This motif is essential for the cell binding activity of this fragment observed in vitro (Pierschbacher and Ruoslahti, 1984). Indeed addition of soluble RGD containing peptides to cultured monolayers leads to cells detaching from the dish, while peptides with mutations in this motif have no effect. A number of subsequent studies have raised the possibility that sequences in repeats 8 and 9 may act synergistically to enhance this adhesion (Aota et al., 1991; Nagai et al., 1991; Yamada et al., 1992). More recently an alternative cell-binding motif has been identified in the CS1 and CS5 alternatively spliced IIICS region of fibronectin. The CS1 site has the minimal active sequence LDV, while the CS5 sequence is REDV, which confers an interaction of lower affinity and specificity (Humphries et al., 1986; Humphries et al., 1987; Mould et al., 1991). These minimal adhesive sequences are far less active than the intact segments implying that other residues also contribute to full adhesive affinity at these regions. For example sequences as far as 14 to 20 kDa away from the RGD central cell binding domain affect the affinity of interactions at this site (Yamada, 1991).

1.2.3 The integrins

The integrins are an evergrowing family of transmembrane glycoprotein cell surface receptors consisting of more than 20 non-covalently bound αβ heterodimers. One or more integrins are expressed on the surface of every type of cell in the body, except for mature erythrocytes (Hemler, 1990). The integrins are known to interact with a variety of factors including ECM components, cell surface Ig superfamily receptors, micro-organisms and a number of plasma proteins. Presently over 14 different α subunits and 8 β subunits have been found in humans by a variety of biochemical and genetic means (Diamond and Springer, 1994). These α subunits are generally only found expressed with one type of β subunit, with the exception of the α5, α4 and α6 subunits, which show more promiscuous expression. The heterodimers formed, the ligand and the cellular expression of particular
heterodimers are summarised in Table 1.2.

The α-subunits
The α subunits range in size from 120-180 kDa, and can be subdivided into three categories on the basis of structural motifs found within the extracellular domains, which consist of 950-1100 amino acids (>100 kDa). Members of one group of α subunits (α1b, α5, α6, α7 and α3) have four putative divalent cation binding sites, each about 60 amino acids long, and a protease cleavage site. The subunit is cleaved at this position and the two fragments linked via a disulphide bridge. Members of the second group (αm, αi, αγ, α1 and α2) have three putative cation binding sites, but lack the protease cleavage site. These subunits also have a 180-200 amino acid insert known as the I domain, not found in any other integrin subunit, which has recently been shown to be a divalent cation binding site (Michishita et al., 1993) that participates in ligand recognition when the subunits are expressed with β2-integrins (Diamond et al., 1993; Michishita et al., 1993; Bisland et al., 1994). The I domain shows homology to domains found in other proteins such as von Willebrand factor, cartilage matrix protein and the complement regulatory proteins, factor B and C2. The third group has only one member, the α4 subunit, which possesses three putative cation binding domains and a protease cleavage site, but lacks the I domain (Takada et al., 1989). The cytoplasmic domains of the α subunits are short (15 to 77 amino acids) and although conserved between species show little homology to each other (Sastry and Horwitz, 1993). They do however all have in common the GFFKR membrane proximal motif thought to be important either for signal transduction or for αβ subunit association (O’Toole et al., 1994). The domain structures representative of each different type of α subunit are modelled in Fig. 1.3A.

The β subunits
The β subunits are smaller than the α subunits ranging in size from 80-120 kDa each possessing similar domain structures. They have an extracellular domain consisting of 675-700 amino acids (>75 kDa), a hydrophobic transmembrane domain and a relatively short cytoplasmic tail of 40-60 amino acids, except β4 which has a cytoplasmic tail of over 1000 amino acids (Hogervorst et al., 1990). Unlike the α subunits the cytoplasmic domains of the β subunits do show homology to each other. The β1, β2, β3, β6 and β7 subunits all show homology in a region, that if deleted in the β2-integrins, affects ligand binding (Hibbs et al., 1991). The N-terminal 40-50 kDa (first 100-200 amino acids) of the β subunit is tightly folded and contributes to the ligand binding domain. A generalised domain structure of the β subunit is shown in Fig. 1.3A. Both integrin subunits exhibit a high level of disulphide bridging (Calvete et al., 1989; Calvete et al., 1991) that helps maintain the structure of the receptor which appears as a globular head comprising both subunits and two stalks extending into the bilayer (Fig. 1.3B). The integrins are not rigid structures, but are conformationally labile and subject to a degree of disulphide bond
Ligand binding

It is regions in the head of the heterodimer that are primarily responsible for ligand binding. Expression of both transmembrane and cytoplasmic sequence deletion mutants of both subunits still results in the formation of functional αβ heterodimers (Bodary et al., 1991; Dana et al., 1991). Ligand binding by a heterodimer not only relies on the respective α and β subunits present, but often also on the co-ordination of cations (Ginsberg, 1995). The nature of these cations can affect both the specificity and affinity of ligand binding, and for some heterodimers are necessary for the association of the two subunits (Galili and Rouslahti, 1988; Kirchhofer et al., 1990; Kirchhofer et al., 1991). A region within the first 100-200 amino acids of the β subunit (Smith and Cheresh, 1988; Smith and Cheresh, 1990), and the cation binding sites of the α subunit appear to be required to form a ligand binding pocket (Fig. 1.2B). Interestingly ligand specificity of particular heterodimers can vary depending on cell type. For example αβ1 expressed in platelets recognises collagen but not laminin, while in other cell types it recognises both ligands (Kirchhofer et al., 1990; Elices and Hemler, 1989).

The role of α and β subunits in heterodimer function

The cytoplasmic tails of both integrin subunits are thought to play important roles in assembly, maturation and localisation of the receptor to sites of adhesions. The integrin heterodimers are mutually interdependent for processing and cell surface expression (Ginsberg, 1995). Indeed downregulating the intracellular pool of β1-integrin by antisense technology increases the maturation rate of remaining β receptor, decreases the maturation rate of the associated α-subunits, and also effects the function of the mature receptor and the ability to assemble the fibronectin fibril network correctly (Koivisto et al., 1994). The cytoplasmic tail of the β subunit appears to be important both for localisation to sites of adhesion, and also for the functional activity of the receptor. The β1-integrin cytoplasmic tail interacts with a number of cytosolic, cytoskeletal proteins namely talin and α-actinin (Horwitz et al., 1986; Otey et al., 1993) and is indeed capable of directing reporter constructs to focal adhesions (LaFlamme et al., 1992), perhaps by interacting with these proteins. Recent studies have also shown that peptides mimicking β-integrin cytoplasmic domains can also bind both pp125Fak and paxillin at residues distinct from those required for binding to α-actinin (Schaller et al., 1995). Clustering deletion mutant β-integrins with antibody coated beads has revealed that the colocalization of talin, pp125Fak and actin with integrins requires residues 791-799 of the β1-integrin tail (Lewis and Schwartz, 1995). Chimaeric reporter constructs have also been used to demonstrate that the cytoplasmic tails of the β subunit can mimic and or inhibit the endogenous receptor. For example a chimera of the β1-integrin cytoplasmic tail fused the the transmembrane and extracellular domains of the interleukin-2 receptor acts has a dominant negative effect on cell spreading and
Fibronectin has a modular mosaic composition common to all extracellular matrix proteins. It is composed of type 1, 2 and 3 repeats that combine to form proteolysis resistant domains that can interact with extracellular matrix proteins as indicated underneath the relevant segment, or with the integrin heterodimers expressed on the cell surface. Two fibronectin molecules may form dimers by forming disulphide bridges at the C-terminus. There are two cell binding domains - the central cell binding domain (CCBD) and the IIICS domain. Within the CCBD is the RGDS motif (represented by arrow 1), that can interact with integrins, but also requires sequences (arrows 2 and 3) in the flanking type 3 repeats for full activity. Similarly, within the IIICS region is the CS1 domain, containing the LDV tripeptide (arrow 4), and the CS5 region containing an RGD-like motif (arrow 5). Arrow 6 represents at least 2 regions that can promote adhesion and interact with heparin. The IIICS region is also able to undergo alternative splicing, generating up to 4 variants. Also, two type 3 repeats - ED-A and ED-B, are subject to alternative splicing. Two disulphide bridges are formed at the C-terminus and serve to cross link fibronectin fibres. (Modified from Ruoshlahti, 1988, Yamada, 1991 and Potts and Campbell, 1994).
<table>
<thead>
<tr>
<th>Integrin</th>
<th>Characterized ligands</th>
<th>Recognition sequence or domain</th>
<th>Distribution</th>
</tr>
</thead>
<tbody>
<tr>
<td>α₁β₁</td>
<td>Collagen I, collagen IV, laminin</td>
<td>DGEA (collagen)</td>
<td>Broad</td>
</tr>
<tr>
<td>α₂β₁</td>
<td>Collagen I, collagen IV, laminin</td>
<td>RGD(?)</td>
<td>Broad</td>
</tr>
<tr>
<td>α₃β₁</td>
<td>Laminin, collagen I, fibronectin, epiligrin</td>
<td>Domain 1 and 4 (VCAM-1) EILDV (CS-1 fibronectin)</td>
<td>Broad</td>
</tr>
<tr>
<td>α₄β₁</td>
<td>VCAM-1, fibronectin</td>
<td>RGD, EILDV</td>
<td>B and T lymphocytes, macrophages, neural crest cells, fibroblasts</td>
</tr>
<tr>
<td>α₅β₁</td>
<td>Fibronectin</td>
<td>RGD</td>
<td>Broad</td>
</tr>
<tr>
<td>α₆β₁</td>
<td>Laminin</td>
<td>Broad</td>
<td></td>
</tr>
<tr>
<td>α₇β₁</td>
<td>Laminin</td>
<td>?</td>
<td></td>
</tr>
<tr>
<td>α₈β₁</td>
<td>?</td>
<td>?</td>
<td></td>
</tr>
<tr>
<td>α₉β₁</td>
<td>Vitronectin, fibronectin</td>
<td>RGD (fibronectin)</td>
<td>Epithelial cells</td>
</tr>
<tr>
<td>α₁β₂</td>
<td>ICAM-1, ICAM-2, ICAM-3</td>
<td>Domain 1 (ICAM-1)</td>
<td>Leukocytes</td>
</tr>
<tr>
<td>α₈β₂</td>
<td>iC3b, ICAM-1, fibronectin, factor X</td>
<td>Domain 3 (ICAM-1), 30kDa plasmin fragment (fibrinogen)</td>
<td>Granulocytes, macrophages, natural killer cells, cytotoxic T lymphocytes</td>
</tr>
<tr>
<td>α₃β₂</td>
<td>Fibrinogen, iC3b</td>
<td>GPRP (fibrinogen)</td>
<td>Macrophages, granulocytes activated B lymphocytes</td>
</tr>
<tr>
<td>α₃β₃</td>
<td>Fibrinogen, fibronectin, vWF, thrombospondin</td>
<td>RGD, KQADGV (fibrinogen)</td>
<td>Platelets</td>
</tr>
<tr>
<td>α₄β₄</td>
<td>Vitronectin, fibronectin, vWF, thrombospondin, fibrin</td>
<td>RGD</td>
<td>Endothelial and tumor cells</td>
</tr>
<tr>
<td>α₅β₄</td>
<td>Laminin (?), basement membrane protein (?)</td>
<td></td>
<td>Epithelial cells</td>
</tr>
<tr>
<td>α₃β₅</td>
<td>Vitronectin</td>
<td>RGD</td>
<td>Carcinoma cells</td>
</tr>
<tr>
<td>α₅β₆</td>
<td>Fibronectin (?)</td>
<td>RGD</td>
<td>?</td>
</tr>
<tr>
<td>α₃β₇</td>
<td>VCAM-1, fibronectin, MAICAM-1</td>
<td>EILDV (fibronectin)</td>
<td>Activated B and T lymphocytes, macrophages,</td>
</tr>
<tr>
<td>α₅β₇</td>
<td>?</td>
<td>Intraspinthelial lymphocytes</td>
<td></td>
</tr>
<tr>
<td>α₅β₈</td>
<td>?</td>
<td>?</td>
<td></td>
</tr>
</tbody>
</table>

**Table 1.2. The integrin superfamily.** (Modified from Diamond and Springer, 1994). Amino acid sequences are denoted by the single-letter code. Abbreviations: VCAM-1, vascular cell adhesion molecule-1; ICAM, intracellular adhesion molecule; iC3b, inactivated complement C3; vWF, von Willebrand factor; MAICAM-1, mucosal addressin cell-adhesion molecule.
Figure 1.3. Schematic representation of individual integrin subunits and a typical integrin heterodimer.

Panel A - The domain structures representative of each of the three classes of integrin α subunits. αIIb is representative of an integrin that contains four cation binding sites and a membrane proximal protease-cleavage site; αM and example of I domain containing integrins; and α4 of its own subclass. β2 is shown as a representative of the β subunits. (Modified from Diamond and Springer, 1994).

Panel B - Represents a simplistic cartoon of the functional heterodimer found at the membrane. The ligand binding site (orange) is formed from the extracellular domains of both subunits. (Modified in part from Hynes, 1992).
migration on fibronectin (LaFlamme et al., 1994). Indeed the cytoplasmic tail of the β3-integrin has been shown to be necessary for cell spreading and focal adhesion formation on fibrinogen (Ylanne et al., 1993). There are examples of such dominant-negative subunits occurring naturally. An alternative spliced variant of the β1 subunit, β1B, possesses a unique cytoplasmic domain, and despite having the same ligand binding properties as the normal subunit, cannot trigger integrin associated signalling events. It also acts in a dominant negative manner on cell adhesion and motility when expressed in CHO cells (Balzac et al., 1994).

The cytoplasmic tail of the α subunit appears to exert functional control on its β subunit partner, but there is little evidence that the α subunit has cytoskeletal associations. Deletion of the cytoplasmic tail of the αIIb subunit results in constitutive, ligand independent, localisation of the β3 subunit to sites of adhesion (Ylanne et al., 1993; Briesewitz et al., 1993). Exchanging the cytoplasmic domain of the α3β1 receptor for those of α3 and α5 conferred functional changes on the heterodimer as a collagen receptor, with collagen binding inducing cellular signals associated with activation of the α3β1 and α5β1 integrins. This implies that α cytoplasmic domains determine the intracellular effects of the various heterodimers formed (Chan et al., 1992). Using chimaeric receptors in which the α and β cytoplasmic domains have been exchanged, reveals that both are required for formation of the heterodimer, and ligand binding, though no post ligand binding events were induced. The expression of the α with the β cytoplasmic domain also prevented ligand independent focal adhesion localisation (Briesewitz et al., 1993; Briesewitz et al., 1995). It is possible that interaction of β with α subunits usually blocks β functional domains, and that ligand occupancy induces α subunits to release this constraint.

Regulation of heterodimer affinity states
Integrin heterodimers can mediate two types of signalling. They can transduce signals by engaging ligand or by receptor clustering, to induce elevation of tyrosine phosphorylation, changes in gene expression and activation of other receptors. This type of signal transduction is known as outside-in signalling. Their affinity for ligands can also be regulated by a process known as inside-out signalling, whereby events at the cytoplasmic membrane proximal region induce conformational changes in the extracellular domain of the receptor. The increase in ligand affinity is a rapid, energy dependent event, which is thought to depend on cytoskeletal elements. It is unlikely that phosphorylation of either subunit facilitates this change. Although the β3 subunit is phosphorylated on serine in response to PKC activators, the stoichiometry is too low to account for the degree of activation (Hillery et al., 1991). The neutrophil β2 subunit also shows phosphorylation in response to certain agonists, but other β subunits activate in the absence of this phosphorylation. Moreover mutating phosphorylation sites has no effect on activation of the receptor (Chatila et al., 1989). Certain α subunits are also phosphorylated, though
again no causal relationship has been established (Ginsberg, 1995). Through constructing chimeric receptors, the cytoplasmic domains of the β subunits have been found necessary to obtain a high affinity state, while mutation of serine 752 to proline of β3 disrupts β3 activation (Chen et al., 1992). In virally transformed fibroblasts, β1 is tyrosine phosphorylated, associated with perturbation of cell adhesion (Hirst et al., 1986), changes in integrin expression and distribution (Johansson et al., 1994), and lowering its association with talin and fibronectin in vitro (Tapley et al., 1989).

Although the signals which convert integrins to a high affinity state have not been defined, two short sequences in the membrane proximal region of the cytoplasmic tails of both subunits are known to play an important role. The first sequence identified was the conserved GFFKR motif in the α cytoplasmic tail, deletion of which locks the heterodimer in the high affinity state for its ligand (O'Toole et al., 1994). This lends further support to the theory that in the absence of an activation signal, the α subunit negatively regulates the β subunit. The second is a similar conserved sequence in the β cytoplasmic tail, the NPXY motif, deletion of which abrogates binding to the ligand even when β tail mutants were expressed with the complimentary mutant α lacking the GFFKR motif (O'Toole et al., 1995). The affinity of the receptor for its ligand (inside-out signalling) therefore appears to be regulated by events at the cytoplasmic face of the heterodimer.

A number of putative integrin regulatory proteins have been found to interact with the cytoplasmic tails of both integrin heterodimers. Calreticulin, a 60 kDa protein, has previously been shown to interact with the (KX)GFFKR motif of integrin α-subunits (Rojiani et al., 1991; Dedhar, 1994), with downregulation of the protein inhibiting cell attachment to the extracellular matrix (Leung-Hagesteijn et al., 1994). It appears to associate with the active high affinity form of the αβ1 integrin possibly maintaining the receptor in the active form once ligand is engaged (Coppolino et al., 1995). A protein known to interact with the cytoplasmic domains of β-integrins is the integrin associated protein (IAP). IAP is a 50 kDa cytoplasmic protein that interacts with the cytoplasmic domain of β3-integrins in a variety of cell types, with antibodies against IAP blocking both β3 mediated ligand binding and integrin-mediated calcium entry into endothelial cells (Calvete et al., 1991; Schwartz et al., 1993). IAP is thought to mediate local calcium fluxes associated with integrin activation events, though its precise role is as yet unknown (Schwartz et al., 1995). One lipid factor, known as integrin modulating factor-1 (IMF-1) has been described that increases the affinity of polymorphonuclear leukocytes αMβ2 integrin for its ligand (Hermanowski-Vosatka et al., 1992). Finally the most recently identified integrin associated protein is the integrin linked kinase, a 59 kDa serine/threonine kinase that interacts with the tail of β1-integrins. Interestingly the activity of the protein is reduced in response to cell adhesion to fibronectin, while overexpression disrupted the cellular architecture of epithelial cells, reduced adhesion to fibronectin, and
increased anchorage independent growth. It is possible that this new kinase plays a role in the negative regulation of integrin function, inactivating the integrin heterodimer (Hannigan et al., 1996).

1.3 Structural cytoskeletal proteins

1.3.1 Talin
Talin is a cytoskeletal protein containing 2541 amino acids with a predicted molecular mass of 270 kDa but an apparent molecular weight of 220 kDa when estimated by gel electrophoresis (Rees et al., 1990). The human talin gene has been mapped to the short arm of chromosome 9 (Gilmore et al., 1995). The domain structure of talin is represented in Fig. 1.4. In vivo talin is distributed between a soluble cytosolic fraction and a detergent insoluble cytoskeletal fraction. Indeed upon activation of platelets it is rapidly recruited to the insoluble cytoskeleton (Beckerle et al., 1989; Bertagnolli et al., 1993a). The calcium-dependent protease calpain II, which has been localised to sites of adhesion (Beckerle et al., 1987), cleaves talin at residues 433 and 434, generating a 47 kDa N-terminal head and a 190 kDa C-terminal tail. Upon thrombin activation of platelets, talin undergoes calpain-mediated cleavage, though the functional significance of this is unknown (Fox et al., 1993). The N-terminal 47 kDa fragment is required for localisation to focal adhesions rather than cell-cell junctions (Nuckolls et al., 1990), and also contains a binding site for charged lipids suggesting that it might facilitate membrane interaction (Dietrich et al., 1993). When normal platelets are activated, talin translocates from the cytoplasm to associate juxtaposed to the membrane (Beckerle et al., 1987). A case for a direct association of talin with the membrane is supported by the observation that this translocation still occurs in the platelets lacking integrin GPIIb-IIIa, the membrane bound binding partner of talin (Bertagnolli et al., 1993a). Within this N-terminal domain, talin shows 23% homology with ezrin, a member of the ezrin/radixin/moesin family which also contains the tumour suppressor NF2/schwannomin and two tyrosine phosphatases PTPH1 and PTPase MEG (Algrain et al., 1993). A strain of rat, Wistar-Furth, that exhibits a high degree of tumour incidence and platelet abnormalities, carries a point mutation in talin at codon 1176 (proline -> threonine), though whether this has functional significance remains to be established (Jackson et al., 1992). As a result, it has been hypothesised that talin possesses tumour suppressor activity.

The 190 kDa region of talin has been shown to contain a number of different binding sites via a variety of in vitro binding studies. Talin interacts with the cytoplasmic tail of β1-integrin (Horwitz et al., 1986), actin (Muguruma et al., 1990), and vinculin (Burridge and Mangeat, 1984). More recently whole talin has been shown to interact with both pp125Fak (Chen et al., 1995) and paxillin (Salgia et al., 1995), though the binding sites have not been defined further. The vinculin-binding sites have been defined in detail with two or possibly three binding sites contained in residues 498-656, 852-950 and 1554-2268. These
sites overlap with sequences known to be required for targeting talin to focal adhesions (Gilmore et al., 1993). Talin has been shown to possess actin nucleating activity, promoting the formation of F-actin filaments from G-actin monomers (Kaufmann et al., 1991) and anchors nucleating actin filaments at lipid membranes (Kaufmann et al., 1992). Talin also enhances the rate of actin crosslinking by α-actinin (Muguruma et al., 1992).

More recently, actin co-sedimentation assays have demonstrated that talin appears to contain a number of actin-binding sites lying within residues 102-497; 952-1328 and 2269-2541 (S.J. Bolton, L. Hemmings and D.R. Critchley, unpublished data). One of these sites lies within the C-terminal residues 2337-2541 which show homology to a yeast actin binding protein Sla2P (Holtzman et al., 1993).

Evidence of the functional importance of talin has come from a number of different sources. It was initially shown that microinjection of a polyclonal antibody against talin inhibited cell migration and cell spreading on fibronectin (Nuckolls et al., 1992). Microinjection of monoclonal antibodies that recognise epitopes within the N- and C-terminal regions of the protein inhibit cell motility and disrupt the integrity of actin stress fibres and focal adhesion in MRC5 human lung fibroblasts (S.J. Bolton, J.M. Wilkinson and D.R. Critchley, unpublished data). Interestingly one group of anti-functional antibodies recognise an epitope in a region which contains one of the recently identified actin binding domains. The role of talin has been more specifically addressed by modifying expression using antisense technology. Reduction of levels of talin expression down to 10% of that of control cells induced a dramatic reduction in the kinetics of HeLa cell spreading. When spread on fibronectin, downregulated cells also exhibited a change in focal adhesion size and distribution, from large peripheral adhesions to smaller adhesions covering the ventral face of the cell. This was also associated with a decrease in the number of stress fibres. The antisense talin expressing cells also exhibited a defect in the ability to regulate and process β1-integrins (Rizo-Albiges et al., 1995).

How talin recruitment to the adhesions is regulated is unclear. It has been reported to be phosphorylated on serine and threonine residues in vitro by PKC (Beckerle, 1990), and within the 47 kDa fragment by a novel kinase, protein kinase P (Simons and Elias, 1993). Talin also exhibits increased phosphorylation in African green monkey kidney (BSC-1) cells (Turner et al., 1989), and in platelets (Bertagnolli et al., 1993a) in response to treatment with phorbol esters, regarded as activators of PKC. Talin is also tyrosine phosphorylated in Rous sarcoma virus (RSV)-transformed fibroblasts (Pasquale et al., 1986) and in cells treated with platelet derived growth factor (PDGF) (Tidball and Spencer, 1993). The functional significance of these phosphorylation events are unclear, but may be associated with the disruption of cell adhesion as talin has not been seen to become tyrosine phosphorylated under conditions of adhesion formation (Bockhold and Burridge, 1993). Interestingly, numerous studies have demonstrated an interaction of talin with...
lipids (Heise et al., 1991; Goldmann et al., 1992; Tempel et al., 1995) leading to speculation that, like a number of other cytoskeletal proteins lipids play a role in regulating talin function. It is possible that the active form of talin may be a homodimer (Goldmann et al., 1994), an observation supported in part by analysis of the repeated motifs within the talin rod (McLachlan et al., 1994). There is also some evidence for a degree of intramolecular interaction within talin. In low salt, the protein is globular and asymmetric as determined by sedimentation equilibrium centrifugation, while at high salt it is more elongated and flexible, observations confirmed by electron microscopic examination of the protein (Molony et al., 1987). This implies that the protein can undergo a degree of intramolecular interaction which may negatively regulate recruitment to the cytoskeleton by masking binding sites within the molecule.

1.3.2 Vinculin

Vinculin is a 116 kDa cytoskeletal protein containing 1066 amino acids, encoded by a single copy gene on human chromosome 10q11.2-qter (Weller et al., 1990). The complete sequence of the human, chick and nematode vinculin and partial sequence of the mouse protein have been published, along with the gene structure of human gene (for review see Hemmings et al., 1995). A muscle specific isoform of vinculin arises due to alternative splicing, resulting in an insertion of an additional 68 amino acids between residues 915 and 916 (Koteliansky et al., 1992). A representation of the domain structure of vinculin is shown in Fig 1.4. When viewed under the electron microscope, vinculin appears to contain a globular head and an extended tail. The head contains the N-terminus of the protein, a putative α-actinin binding site (residues 1-107) (Wachsstock et al., 1987; Kroemker et al., 1994), a talin binding site (residues 1-258) (Burridge and Mangeat, 1984; Gilmore et al., 1992), and three 112-residue repeats of unknown function. The head can be liberated from the tail (30 kDa) as a 90 kDa fragment by V8 protease cleavage within the proline rich region. The C-terminal region contains a paxillin binding site (residues 978-1000) (Wood et al., 1994), and an F-actin binding site (residues 893-1016) (Menkel et al., 1994). Vinculin has also been shown to bind to the actin-capping protein tensin, though the binding site within vinculin has not been further defined (Wilkins and Lin, 1986). The associations vinculin makes with α-actinin, talin, paxillin, tensin and F-actin suggests that multiple pathways exist for the linkage of integrins to the actin cytoskeleton.

Vinculin undergoes an intramolecular association between the N-terminal head and the C-terminal tail domains (Kroemker et al., 1994; Johnson and Craig, 1995a), which is likely to regulate recruitment to adhesions. The head-binding site in the vinculin tail has been mapped to between residues 1029 and 1036 using deletion mutants, and the tail-binding site in the head to within residues 1-258 (Weckes et al., 1996). The interaction between the head and tail has been shown to modulate talin (Johnson and Craig, 1994), α-actinin (Kroemker et al., 1994) and F-actin (Johnson and Craig, 1995a) binding to vinculin. The
head-tail interaction is relieved in the presence of acidic phospholipids revealing the actin-binding site and sites for PKC phosphorylation in the C-terminal tail (Weekes et al., 1996). Vinculin has been shown to contain a cryptic binding site for acidic phospholipids (Johnson and Craig, 1995b), and also to co-precipitate with the acidic phospholipid phosphotidylinositol bisphosphate (PIP2) (Fukami et al., 1994). Indeed vinculin can form a ternary complex with both α-actinin and phospholipids in vitro (Niggli and Gimona, 1993), and an in vivo association with the membrane has been demonstrated in chicken embryo fibroblasts using photoactivated fatty acids (Niggli et al., 1990). In vivo vinculin is phosphorylated on tyrosine residues in cells transformed with RSV (Pasquale et al., 1986), and in a calcium-dependent manner in stimulated platelets (Vostal and Shulman, 1993). It has also been shown to be a substrate for PKC in vitro (Werth et al., 1983) and also to show increased serine and threonine phosphorylation upon treatment of chick embryo and Swiss 3T3 fibroblasts with phorbol esters (Werth and Pastan, 1984). The stoichiometry of this phosphorylation is low, although early evidence suggested that the phosphorylated protein was predominantly associated with the insoluble cytoskeletal fraction (Geiger, 1982).

A number of studies have demonstrated the functional importance of vinculin in cell adhesion. Microinjection of an anti-vinculin antibody into cultured fibroblasts results in the disruption of actin stress fibres and focal adhesions (Westmeyer et al., 1990). Overexpression of vinculin in Balb/c 3T3 cells was found to reduce motility but increase adhesion (Fernandez et al., 1992a). Conversely, reduction of the levels of vinculin by constitutive expression of antisense RNA was found to reduce the ability of cells to spread on fibronectin and increased motility. Down-regulated cells were also able to grow in soft agar suggesting that downregulation of vinculin contributes to a transformed phenotype (Fernandez et al., 1993). However down-regulation of vinculin by antisense in Balb/c 3T3 using inducible expression of the antisense RNA does not confer cells with the ability to grow in soft agar. Indeed these cells possessing reduced vinculin show reduced growth rates in comparison to control cells, and when spread on fibronectin do not show increased tyrosine phosphorylation of pp125Fak or paxillin (S.J. Bolton, S.T. Barry and D.R. Critchley, unpublished data). The vinculin gene has been disrupted by chemical mutagenesis in a mouse F9 embryonic carcinoma cell line. In agreement with the antisense experiments, these cells exhibited a rounded morphology and were defective in both cell-cell and cell-matrix adhesion. The adhesive phenotype was restored by introduction of a plasmid encoding a full length chick-vinculin (Volberg et al., 1995). Expression of vinculin in a SV40 transformed cell line expressing one quarter of the levels of vinculin present in untransformed cells suppresses the transformed phenotype (Fernandez et al., 1992b). Like talin, this suggests a role for vinculin as a possible tumour suppressor.
1.3.3 α-actinin

The rod shaped protein α-actinin forms an anti-parallel homodimer with a subunit molecular mass of approximately 100 kDa. The domain structure of the protein is shown in Fig. 1.4. It is an F-actin crosslinking and bundling protein found at sites where actin is linked to a variety of intracellular structures, including focal adhesions (Blanchard et al., 1989). At least three α-actinin genes have been identified, one of which encodes the smooth muscle isoform plus a non-muscle isoform that arises via alternative splicing of an exon encoding part of the first EF-hand calcium binding motif (Waites et al., 1992). This may account for the different calcium sensitivities of the two isoforms with regard to actin binding (Witke et al., 1993). Alternative splicing of the chick skeletal-muscle α-actinin gene has also been observed (Parr et al., 1992). Interestingly α-actinin isolated from rabbit macrophages has been shown to bind actin in a calcium-independent manner, contradicting the established view that non-muscle α-actinins are calcium-dependent actin-binding proteins (Pacaud and Harricane, 1993). A combination of biochemical work in vitro and in vivo rescue studies in Dictyostelium discoideum with α-actinin mutants have shown that only the first EF-hand calcium binding domain plays a role in regulating actin binding (Witke et al., 1993).

α-Actinin binds F-actin via its N-terminal domain (residues 1-266), with one actin binding site being localised to residues 120-134 within the chick smooth muscle protein (Hemmings et al., 1992; Kuhlman et al., 1992; Kuhlman et al., 1994). The homologous N-terminal domains within dystrophin (Hemmings et al., 1992) and utrophin (Winder et al., 1995) are also capable of binding actin. The N-terminal domain of α-actinin also binds to zyxin, an 82 kDa LIM domain-containing protein of unknown function which also localises to focal adhesions (Crawford et al., 1994). Dimerisation of the α-actinin molecule is mediated by the central region of the molecule that consists of four α-helical spectrin-like repeats. The boundaries between these repeats has recently been revised on the basis of limited proteolysis studies with bacterially expressed proteins (Gilmore et al., 1994). Chemical crosslinking and sedimentation equilibrium analysis has revealed that repeats 1 and 4 interact to bring about dimerisation (Flood et al., 1995). α-Actinin has been shown to interact with vinculin, with the binding site lying between residues 713-749 (Wachstock et al., 1987; McGregor et al., 1994). The repeat region has also been shown to be the site at which α-actinin interacts with β1-integrin (Obey et al., 1993). Titin and α-actinin have been shown to interact, with α-actinin binding to the A-domain of titin (Wang and Jeng, 1992). An interaction between α-actinin and the intercellular cellular adhesion molecule-1 (ICAM-1) has been detected in vitro (Carpen et al., 1992).

A variety of techniques have been used to analyse the functional role of α-actinin in vivo. Microinjection of a 27 kDa actin-binding fragment and a 53 kDa fragment corresponding to the repeat region, produced by thermolysin proteolysis of α-actinin, disrupted both actin
**Figure 1.4. Domain structures of talin, vinculin and α-actinin.**
stress fibres and focal adhesions at high concentrations (Pavalko and Burridge, 1990). Overexpression of α-actinin in Balb/c 3T3 fibroblasts by up to 40-60% higher than normal reduced cell motility. Conversely downregulation of α-actinin, by antisense, to between 25-60% of that in normal cells increased cell motility (Gluck and Ben-Ze’ev, 1994). Like vinculin, overexpression of α-actinin in SV40 transformed cells reduced tumorgenieity, further evidence for the possible role of the cytoskeletal proteins as tumour supressor proteins (Gluck et al., 1993). Genetic analysis of the functional importance of α-actinin in vivo has been probed by homologous recombination in D. discoideum. Deletion of α-actinin alone did not result in a phenotypic change. However ablation of a second actin-crosslinking protein ABP-120 produced slime moulds that were normal apart from being unable to form fruiting bodies (Witke et al., 1992). In addition several α-actinin Drosophila null mutants have been identified that possess only minor muscle defects (Roulier et al., 1992). This highlights the functional redundancy apparent between members of the actin-cross-linking family of proteins.

How recruitment of α-actinin to adhesive structures is regulated is unclear. It has been shown to interact with PIP2 in vivo (Fukami et al., 1994), an interaction known to augment the actin cross-linking activity of the protein in vitro (Fukami et al., 1992). The actin gelation activity of talin is increased upon the formation of a complex with α-actinin (Muguruma et al., 1992). As discussed earlier α-actinin can form a ternary complex in vitro with vinculin and acidic-lipid bilayers (Niggli and Gimona, 1993), in which both interact with the bilayer. It is possible that in concert with the activating action of acidic phospholipids, α-actinin plays a role in regulating the activity of other cytoskeletal proteins. Little evidence is available in the literature regarding the phosphorylation of α-actinin.

1.3.4 Zyxin and cCRP
Zyxin is a low abundance 82 kDa protein discovered as an antigen recognised by antibodies in a non-immune rabbit serum that stained focal adhesions (Beckerle, 1986). The protein was initially purified from avian smooth muscle (Crawford and Beckerle, 1991), and subsequently shown to interact with α-actinin, thought to localise the protein to focal adhesions (Crawford et al., 1992). Cloning of chicken zyxin revealed that the N-terminus contained an array of proline residues while the C-terminus contained three tandemly arrayed LIM motifs, capable of binding zinc (Sadler et al., 1992). Screening for zyxin binding partners by gel overlay revealed that, as well as α-actinin, it interacts with a second 23 kDa LIM domain containing protein the (chicken) cysteine-rich protein (cCRP) which also localised to focal adhesions (Sadler et al., 1992). cCRP is 91% identical to the human CRP (hCRP), contains two LIM domains, and has been shown to be developmentally regulated, with expression increasing in the latter stages of development (Crawford et al., 1994). Like zyxin the cCRP LIM domains are also capable of binding
zinc, which is thought to stabilise interactions made by LIM domains. Indeed one of the three LIM domains in zyxin has been shown to be sufficient and necessary to support an interaction between zyxin and cCRP (Schmeichel and Beckerle, 1995). Recently a zyxin-related protein has been cloned and shown to interact with VASP. This 83 kDa protein was purified from porcine platelets. Sequence analysis suggests that this protein represents the mammalian equivalent of zyxin, and is thought to play a role in localising VASP to focal adhesions (Reinhard et al., 1995).

The functions of zyxin and cCRP are unknown. As discussed above LIM domains are typically found in the N-terminal region of transcription factors, and mediate protein-protein interactions essential for the functional regulation of transcription factors. One hypothesis is that LIM domains regulate process such as cell differentiation and embryonic development by regulating gene expression, in a manner that involves interaction with transcription factors. However neither protein has been shown to have a nuclear location, under normal circumstances. Alternatively the LIM domains may mediate protein-protein interactions required to localise the zyxin to specific cellular locations.

1.4 Tyrosine kinase substrates associated with focal adhesions
(refer also to Fig. 1.9)

1.4.1 Structural domains implicated in adhesion related signalling events

Interactions between members of signalling pathways are often mediated by specialised conserved domains. These domains, termed the src homology 2 (SH2) and src homology 3 (SH3) domains, function in many tyrosine kinase signalling pathways. These domains have been implicated in playing a role in regulating interactions between proteins involved in adhesion regulation. The structure and function of these domains will be discussed briefly.

SH2 domains

SH2 domains were originally identified as regions of homology shared between the non-catalytic domains of src-related kinases spanning approximately 100 amino acids (Sadowski et al., 1986), and have subsequently been identified in numerous proteins. SH2 domains are found in a number of proteins which may play a role in mediating adhesive responses including tensin, csk, crk, grb2, vav, the p85 subunit of PI 3-kinase, and a number of tyrosine phosphatases (Cantley and Songyang, 1994). Autophosphorylation of receptor tyrosine kinases often creates binding sites for the recruitment of specific substrate proteins via interaction with their SH2 domain. For example, tyrosine phosphorylation of the PDGF-receptor at tyrosine residues 1021 and 1009 creates binding sites for the SH2 domains of PLC-γ1 and syp; while tyrosine phosphorylation at residue 771 within the kinase insert domain facilitates binding of rasGAP. Phosphorylation at tyrosines 740 and 751 supports binding of the two SH2 domains of the p85 subunit of PI 3-kinase; while
phosphorylation of tyrosine 579 and 581 allows binding of c-src (Pawson and Schlessinger, 1993). SH2 domains therefore recognise specific tyrosine phosphorylated residues within short peptide sequences, with the three amino acids immediately C-terminal of the tyrosine determining the type of SH2 domain bound (Songyang et al., 1993). The various SH2 domains have been divided into five sub-groups based on the preferred phosphopeptide consensus sequence targeted by the SH2 domain (Songyang et al., 1993; Cantley and Songyang, 1994). Significantly many cytoskeletal proteins contain tyrosine residues which lie within the correct consensus sequences to act as SH2 binding sites. Crystallisation of the SH2 domain of src has revealed that it resembles a two holed socket into which the tyrosine phosphorylated peptide inserts (Waksman et al., 1993). The phosphate binding pocket is highly conserved with the variation between SH2 domains occurring in the second hydrophobic pocket. It is the nature of these three dimensional interactions that confers the specificity on the interaction between a given SH2 domain and its target phosphopeptide sequence (Kuriyan and Cowburn, 1993).

SH3 domains
Like SH2 domains, SH3 domains were again discovered in the src family kinases (Mayer et al., 1988), and were subsequently found in many other proteins that contain SH2 domains. Although much interest has been directed towards defining the properties of the SH2 domains, the significance of the SH3 domains are only just becoming clear. SH3 domains appear to act to localise proteins to specific subcellular locations by directing protein-protein interactions (Bar-Sagi et al., 1993). They are approximately 60-80 amino acids in size (Pawson and Gish, 1992) and like the SH2 domains appear to fall into different classes conferred by structural variations. The SH3 domains of a number of proteins have been studied by both solution NMR (Yu et al., 1992; Booker et al., 1993; Kohda et al., 1993; Koyama et al., 1993) and by X-ray crystallography (Noble et al., 1993) in the presence and absence of ligand. The three dimensional structure of the SH3 domain differs from that of the SH2 domain in that two anti-parallel β-sheets distribute to localise a number of conserved hydrophobic residues together, which probably forms the ligand binding site (Kuriyan and Cowburn, 1993). The SH3 domain-binding motif was identified as a proline-rich sequences of around 10 amino acids in length (Ren et al., 1993). The consensus sequence for SH3 binding has been defined to be XPXXPPΨXPX (where Ψ represents a hydrophobic residue) (Williamson, 1994). Potential SH3 binding sites have been identified on a number of cytoskeletal proteins, but how the interactions made by SH3 domains is regulated is unclear.

1.4.2 Tensin
Tensin is a 215 kDa actin-binding and capping protein originally identified as a contaminant of early vinculin preparations (Wilkins and Lin, 1986; Bockholt and Burridge, 1992). It is thought to form a dimer of 400 kDa in vivo enabling it to interact with two
actin filaments (Lo and Chen, 1994). Antibodies against tensin have localised the protein to both focal adhesions and myotendinous and neuromuscular junctions, supporting the theory that it plays a role in anchoring actin filaments to the cell membrane (Glenney and Zokas, 1989; Bockholt et al., 1992). The complete chick tensin sequence of 1744 amino acids has been published (Davis et al., 1991). The sequence revealed that tensin shares a region of homology with a weak actin-capping protein insertin. Indeed the sequence identity is so close that insertin is likely to be a proteolytic fragment of tensin (Lo and Chen, 1994). Insertin caps actin filaments in a manner that allows addition (or insertion) of actin monomers to the barbed end of the filament at a reduced rate (Wilkins and Lin, 1986), a property also possessed by tensin (Lo and Chen, 1994). Tensin also binds along actin filaments, possessing three distinct actin-binding domains at residues 1 to 263, 263-463 and 888-989, the latter being the region with homology to insertin (Lo et al., 1994). Tensin may play a role in anchoring growing actin filaments to the focal adhesion. By binding actin and other cytoskeletal proteins such as vinculin, tensin may integrate the filament into the focal adhesion (Wilkins et al., 1987), while controlling addition of actin monomers to the barbed end.

Tensin also contains an SH2 domain (Davis et al., 1991). A fusion protein corresponding to the SH2 domain of tensin coupled to agarose beads was used to screen lysates of normal and RSV transformed Rat-1 cells for binding partners. A large number of proteins were precipitated by this peptide, with the amount increased in the transformed cells. These proteins had molecular weights of 160, 120-130, 95, 90, 70 to 80, 66 and 60 kDa (Davis et al., 1991), though whether all of these protein interact directly with the tensin SH2 domain is unlikely. Some of these proteins are likely to have co-precipitated complexed to proteins interacting specifically with the tensin peptide. Specific interactions have been demonstrated between the tensin SH2 domain and both src and paxillin (Lo et al., 1994).

Examination of the pattern of phosphorylation of tensin reveals that it is phosphorylated on both tyrosine and serine/threonine residues. The tyrosine phosphorylation of tensin is low in normal cultured cells but is increased upon transformation by RSV (Davis et al., 1991), and most significantly when cells are adhering and spreading on fibronectin (Bockholt and Burridge, 1993) or when cells are stimulated with angiotensin II (Turner et al., 1995). This tyrosine phosphorylation of tensin may result from the action of either pp125Fak or src family members which phosphorylate the protein in vitro, and may serve to create binding sites for other proteins. Tensin has also been reported to be a substrate for PKC and cdc 2 kinase in vitro (Lo et al., 1994a; Lo and Chen, 1994). The functional significance of these phosphorylation events is unknown. Like a number of other cytoskeletal proteins, overexpression of tensin in transformed cells reduces the tumorigenicity of the cells (Lo et al., 1994a). The domain structure of tensin is represented in Fig. 1.5.
Figure 1.5. **Domain structure of tensin.** Representation of the known binding domains and the putative vinculin binding site. The region thought to correspond to the protein insertin is indicated.
1.4.3 Paxillin

Paxillin is one of a number of proteins (which includes tensin) discovered by generating a panel of monoclonal antibodies against proteins tyrosine phosphorylated in RSV transformed cells (Glemiey and Zokas, 1989). It is a 68 kDa protein which migrates as a diffuse band on SDS-PAGE due to multiple phosphorylations on tyrosine, and serine residues (Schaller and Parsons, 1995). The isoforms have pIs ranging from 6.31 to 6.85 (Turner et al., 1990). Paxillin has been shown to be expressed in a wide variety of tissues, being most abundant in muscle tissue, though levels are reduced in skeletal and cardiac muscle, while no paxillin protein has been detected in either neuronal tissue or platelets (Turner et al., 1990). However paxillin mRNA is expressed in brain (Salgia et al., 1995). The absence of the protein in platelets is somewhat surprising as platelets possess many of the attributes of focal adhesions, and possess high levels of other cytoskeletal proteins (Turner, 1994), suggesting that paxillin serves some other function than purely mediating the adhesive response. Interestingly paxillin expression is high during the early stages of development, while expression is relatively low in adult tissues (Turner et al., 1993). It is also one of the major tyrosine phosphorylated proteins in the developing embryo (Turner, 1991). At the subcellular level paxillin is localised to smooth muscle dense plaque and the myotendinous and neuromuscular junctions of skeletal muscle (Turner et al., 1991), as well as localising to focal adhesions, but is absent from the zonula adherens cell-cell junction of MDCK cells (Turner et al., 1990).

Paxillin cDNAs have been cloned from human, mouse and chicken (Turner and Miller, 1994; Salgia et al., 1995), and the human gene localised to chromosome 12q24 (Salgia et al., 1995). A representation of the domain structure of the protein is shown in Fig. 1.6. At the C-terminus paxillin contains four LIM domains, which are also present in two other cytoskeletal proteins zyxin and the cysteine rich protein (cCRP) (Sadler et al., 1992). This may indicate that paxillin co-ordinates signalling complexes assembled within particular regions of the cell perhaps regulating gene expression. Both paxillin and zyxin are unique within this "family" of LIM domain-containing proteins in that their LIM domains are within the C-terminal region, rather than the at N-terminal end (Turner and Miller, 1994). The LIM domains found in paxillin, zyxin and cCRP may directly interact with transcription factors recruiting them to the correct signalling apparatus. The far N-terminal region of paxillin contains a short proline rich motif that corresponds to a potential SH3-binding domain (Ren et al., 1993), which has been shown to bind to src (Weng et al., 1993), possibly providing one mechanism by which src can localise to focal adhesions.

From analysis of the amino acid sequence a number of tyrosine residues have been identified that if phosphorylated would create potential binding site for the SH2 domains of src family members (tyrosine 35 and tyrosine 485), v-crk (tyrosine 113), phospholipase Cγ (tyrosine 77), and the p85 subunit of PI 3-kinase (tyrosine 431) (Turner and Miller, 1994).
Through such interactions paxillin may serve as an adaptor protein to integrate different regulatory elements. Various stimuli have been shown to induce extensive tyrosine phosphorylation of paxillin, namely, neuropeptides (Zachary et al., 1993), LPA (Barry and Critchley, 1994; Seufferlein and Rozengurt, 1994), PDGF (Rankin and Rozengurt, 1994), angiotensin II (Turner et al., 1995) and fibronectin (Burrige et al., 1992), while in haematopoietic cells it is phosphorylated in response to interleukin-3 and several other haematopoietic cytokines (Salgia et al., 1995). In addition to putative tyrosine phosphorylation sites a number of serine and threonine residues are potential sites for phosphorylation by PKC (Turner and Miller, 1994), a signalling protein required in addition to tyrosine kinases for cell spreading and focal adhesion formation (Woods and Couchman, 1992; Vuori and Ruoslahti, 1993).

Paxillin has been identified as an in vitro substrate for a number of adhesion associated tyrosine kinases, pp125Fak, src and csk (C-terminal src kinase, the negative regulator of src) (Schaller and Parsons, 1995; Bellis et al., 1995). Tyrosine phosphorylation of paxillin by pp125Fak at residues 31 and 118 creates a high affinity binding site for the adapter molecule v-crk (Schaller and Parsons, 1995). An in vivo association of v-crk with paxillin was previously demonstrated by co-immunoprecipitation of the two proteins from cell transformed with CT10 virus (Birge et al., 1993). Paxillin also forms a complex with csk and crk (Sabe et al., 1994), an interaction mediated via SH2 domains (Schaller and Parsons, 1995). pp125Fak requires the focal adhesion targeting (FAT) sequence to tyrosine phosphorylate paxillin. This may however be because FAT region overlaps with the paxillin binding site in pp125Fak, so deletion of the FAT sequence may prevent pp125Fak interacting with paxillin (Hiklebrand et al., 1995). Paxillin is also tyrosine phosphorylated in vitro by the oncoprotein p210BCR/ABL and has been shown to co-immunoprecipitate with it (Salgia et al., 1995), indicating the formation of a complex between paxillin and p210BCR/ABL.

A number of other paxillin binding partners have been identified by a combination of co-immunoprecipitation and in vitro binding assays. As mentioned above, tyrosine phosphorylated paxillin binds the SH2 domains of v-crk and csk, and the SH3 domain of src. Vinculin binds to paxillin within a region towards the N-terminus of paxillin (residues 56-113) (Turner et al., 1990; Turner and Miller, 1994), which has recently been further defined to residues 56-100 (Salgia et al., 1995). The pp125Fak binding site within paxillin is yet to be defined further (Turner and Miller, 1994). A possible interaction of paxillin with talin has also been demonstrated within residues 100-227 (Salgia et al., 1995) though detection of this interaction is not consistent between groups (Turner and Miller, 1994). Using peptides containing the C-terminus of β-integrin cytoplasmic domains an interaction has been detected between β1-integrin and paxillin (Schaller et al., 1995). Little is known about the functional role of paxillin. It is thought that it may function as an adapter
Paxillin

Figure 1.6. The domain structure of paxillin. Representation of the known binding domains of paxillin, the site of interaction with bcr/abl is not known.
molecule mediating interactions between certain signalling molecules and the cytoskeleton. By virtue of its interaction with the oncogenic adapter protein v-crk, paxillin may play a role in regulating small GTP-binding proteins and ultimately the actin cytoskeleton. v-crk contains two SH2 and one SH3 domain, and can interact with the guanine nucleotide exchange factors C3G and SOS which both promote the GTP bound form of p21ras (Matsuda et al., 1994; Tanaka et al., 1993). Paxillin may utilise this to control the activation of GTP-binding proteins with in specific regions of the cell.

1.4.4 Other major proteins tyrosine phosphorylated during the adhesive response
When fibroblasts mount an adhesive response either by spreading on fibronectin or upon stimulation of serum-starved cells with lysophosphatidic acid (LPA), a number of proteins of unknown function are also tyrosine phosphorylated. Three of these proteins of 130 kDa, 110 kDa and 80/85 kDa have been cloned and are named pp130cas, pp110 actin filament associated protein (p110AFAP) and cortactin respectively.

pp130cas
pp130cas is heavily tyrosine phosphorylated in response to adhesion-related stimuli (fibronectin or LPA/endothelin) and transformation of cells by RSV (Vuori and Ruoslahti, 1995; Petch et al., 1995; Nojima et al., 1995; Sosseleben and Rozengurt, 1994; Hordijk et al., 1994; Saville et al., 1994; Kanner et al., 1990). In fibroblasts, pp130 distributes along the stress fibres and also concentrates at sites of adhesion, a staining pattern reminiscent of α-actinin (Petch et al., 1995). It exhibits the same kinetics of tyrosine phosphorylation as pp125Fak when cells spread on fibronectin (Nojima et al., 1995). In epithelial cells pp130cas has also been localised to cell-cell junctions (Reynolds et al., 1994). Immune-complex kinase assays of precipitated pp130 indicate that it was not itself a kinase (Petch et al., 1995) although it has been shown to interact with the SH2 domains of both v-src and v-crk (Reynolds et al., 1989; Kanner et al., 1990), with stable association requiring both the SH2 and SH3 domains of src and crk (Kanner et al., 1991). Cloning and characterisation of pp130cas revealed that it possesses a single SH3 domain, a cluster of SH2-binding motifs, but shows no homology to any known kinase (Sakai et al., 1994). Upon adhesion to the ECM, pp130cas has been shown to translocate from the cytoplasm to the plasma membrane as a result of tyrosine phosphorylation, further evidence for a role in mediating adhesive responses (Vuori and Ruoslahti, 1995; Nojima et al., 1995). Nothing is as yet known about the function of pp130, but from the sequence data, it seems likely that it may function as another adapter molecule mediating cellular signalling events.

pp110AFAP
Like pp130cas, pp110AFAP was originally identified as one of the proteins tyrosine phosphorylated in cells transformed by RSV (Kanner et al., 1990), and as a protein that co-immunoprecipitated with both v-src and c-src (Reynolds et al., 1989). In fibroblasts
Pll OAFAP shows punctate staining along the actin filaments (hence its name actin filament associated protein) and it is also found in podosomes formed upon transformation with RSV (Flynn \textit{et al}, 1993). The stable interaction of p110AFAP with src proteins requires both the SH2 and SH3 domains of src, though tyrosine phosphorylation by src can be achieved with only a functional SH3 domain (Kauner \textit{et al}, 1991). From sequence analysis, p110AFAP contains consensus binding sites for both SH2 and SH3 (2) domains. As well as being tyrosine phosphorylated it appears to be normally phosphorylated on both serine and threonine residues, and indeed contains consensus sequences for phosphorylation by PKC (Flynn \textit{et al}, 1993). More recently a variant form of p110AFAP has been cloned, named p120AFAP. This is a 120 kDa protein expressed only in brain, with a 258 bp insertion, which supports an association with an unidentified 67 kDa protein through a proline-rich motif (Flynn \textit{et al}, 1995). The function of p110/120 and the mechanism of association with the actin cytoskeleton is unknown, but by virtue of its consensus sequences for SH2 and SH3 binding domains it may be an adapter protein recruiting signalling proteins to the cytoskeleton.

\textbf{Cortactin}

Cortactin is an 80/85 kDa protein which, as its name suggests, is responsible for organising the cortical actin network, and is enriched in cortical structures, such as membrane ruffles and lamellapodia (Wu and Parsons, 1993). It migrates as two species in SDS-PAGE, (80 kDa and 85 kDa) and appears to be phosphorylated on serine and threonine residues as well as tyrosine. Hyperphosphorylation of cortactin by c-src is associated with disruption of actin filaments in fibroblasts isolated from csk null mice, while subsequent knockout of c-src restores the cytoskeletal organisation (Thomas \textit{et al}, 1995). Cortactin becomes tyrosine phosphorylated when cells mount an adhesive response or are stimulated with certain growth factors, but this does not appear to effect its actin binding properties. This tyrosine phosphorylation of cortactin correlates well with the kinetics of pp125Fak phosphorylation and the formation of actin filaments (Vuori and Ruoslahti, 1995; Wu and Parsons, 1993). It associates with c-src and is similarly tyrosine phosphorylated in late G1 when Balb/c 3T3 are stimulated by prolonged exposure fibroblast growth factor1 (FGF-1) (Zhan \textit{et al}, 1993a; Zhan \textit{et al}, 1993b; Zhan \textit{et al}, 1994). Cloning of cortactin reveals that it consists of an N-terminal series of six, 37 amino acid tandem repeats, with a C-terminal SH3-like domain (Wu \textit{et al}, 1991). The actin-binding site has been localised to the amino terminal repeat region (Wu and Parsons, 1993). Perhaps of most significance is the cloning of a human cortactin gene identified independently in a number of human tumours. The product of the \textit{EMS}I gene is 85% identical to the known cortactin sequences and has the same domain structure (Schuuring \textit{et al}, 1993). This gene is frequently co-amplified and overexpressed in human breast cancer and in squamous cell carcinomas, and is associated with a more invasive carcinoma (Schuuring \textit{et al}, 1992a; Schuuring \textit{et al}, 1992b). Within the transformed cells, cortactin is associated with podosomes rather than
having a more peripheral distribution, leading the authors to hypothesise that overexpression may contribute to the invasive potential of these cells (Schuuring et al., 1993).

1.5 Protein kinases associated with the regulation of cell adhesion
(refer also to Fig. 1.8)

1.5.1 pp125Fak

Like paxillin, pp125Fak was initially identified as one of a number of proteins tyrosine phosphorylated in v-src transformed chick cells, which localised to focal adhesions (Kanner et al., 1990). The pp125Fak sequence revealed that it was a novel cytoplasmic tyrosine kinase, with a central catalytic domain (Schaller et al., 1992). pp125Fak bears no homology to any other known kinase outside the kinase domain. pp125Fak homologues have been identified in mouse, human and Xenopus (Parsons et al., 1994). The murine mRNA (4.5 kb) is expressed in most cell lines and tissues examined (Hanks et al., 1992; Schaller and Parsons, 1994), though not in some B- and T-cell populations, while macrophages and mast cells express little pp125Fak (Choi et al., 1993; Whitney et al., 1993). Comparison of the mouse and human sequence reveals a high degree of amino acid sequence identity (94% overall and up to 98% within the kinase domain) suggesting a highly conserved function for the protein. The human sequence contains a 29 residue insert within the kinase domain, a feature not shared with homologues from other species. A human pp125Fak brain specific isomer has also been cloned that lacks the N-terminal 157 residues (Andre and Backer-Andre, 1993). In rodent and chick, a smaller 41/43 kDa protein corresponding to the non-catalytic C-terminus of pp125Fak, named Fak-related-nonkinase (FRNK), has been found to be autonomously expressed in some cells. This is thought to arise from alternative splicing of the full length message to give a 2.4 kb mRNA. A similar size mRNA has been detected in human tissues but has not been confirmed as being FRNK. Like pp125Fak, FRNK localises to focal adhesions, but is not tyrosine phosphorylated, though it does become hyper (serine/threonine) phosphorylated as pp125Fak becomes tyrosine phosphorylated (Hanks et al., 1992; Andre and Backer-Andre, 1993; Schaller et al., 1993). Domain structures of both pp125Fak and FRNK are represented in Fig. 1.7.

A second member of the pp125Fak subfamily of tyrosine kinases has been cloned from rat (Sasaki et al., 1995). This protein, named Cell Adhesion Kinase β (CAKβ), is a 115.7 kDa (predicted, 113 kDa by immuneprecipitation) protein tyrosine kinase with N- and C-terminal domains of 418 and 330 residues flanking a central kinase domain. CAKβ shows homology with pp125Fak over the entire length except for the N-terminal 88 residues (45% sequence identity, 60% in the catalytic domain), indicating that CAKβ is structurally related but distinct from pp125Fak. The expression of CAKβ is more restricted than that of pp125Fak in terms of tissues and cell lines. CAKβ is tyrosine phosphorylated in a
manner that is not reduced upon trypsinisation, and not enhanced upon spreading of 3Y1 fibroblasts on fibronectin. Expression of the CAKβ cDNA in COS-7 cells resulted in localisation to cell-cell junctions, and could possibly participate in the signal transduction regulated by cell-cell adhesion (Sasaki et al., 1995).

Phosphorylation of pp125Fak

pp125Fak localises to the myotendinous junctions of smooth muscle in *Xenopus* tissue (Baker et al., 1994). Expression has been shown to be regulated during development of chick embryos, and changes in tyrosine phosphorylation occur as development proceeds (Turner et al., 1993). Indeed pp125Fak has been shown to increase in tyrosine phosphorylation when cells are stimulated with a variety of factors. Stimulation of a number of fibroblast cell lines with bombesin, endothelin, PDGF, LPA and bradykinin all induce rapid tyrosine phosphorylation of pp125Fak, often associated with both the tyrosine phosphorylation of paxillin and the formation of focal adhesions (Sinnett-Smith et al., 1993; Barry and Critchley, 1994; Hordijk et al., 1994; Leeb-Lundberg et al., 1994; Rankin et al., 1994; Ranlein and Rozengurt, 1994; Saville et al., 1994; Seufferlein and Rozengurt, 1994). It is also tyrosine phosphorylated when fibroblasts spread on fibronectin (Burrage et al., 1992; Guan and Shalloway, 1992; Schaller et al., 1993), and when monocytic cells are stimulated by integrin-mediated adhesion (Lin et al., 1995). pp125Fak has been shown to be tyrosine phosphorylated in activated platelets in a manner that is dependent upon the ability of platelets to aggregate. The enzymatic activity of pp125Fak is not elevated in platelets isolated from patients with Glanzmann's thrombasthenia which lack functional αIIbβ3 required for platelet activation. (Liptart et al., 1992). Tyrosine phosphorylation of pp125Fak is also inhibited if the cytoskeleton of platelets or fibroblasts is disrupted by treatment with cytochalasin D (Liptart et al., 1992; Sinnett-Smith et al., 1993; Rankin and Rozengurt, 1994; Seufferlein and Rozengurt, 1994). pp125Fak kinase activity appears to be dependent upon the integrity of the cytoskeleton, and on aggregation of integrins. Both pp125Fak and FRNK are substrates for serine/threonine kinases, and both show increased phosphorylation on serine residues when cells spread on fibronectin (Schaller et al., 1993; Hatai et al., 1994) though which kinases are involved is not known.

Src and fyn have been shown to stably associate with pp125Fak in vitro and in vivo (Cobb et al., 1994). Both proteins interact with pp125Fak as a result of an autophosphorylation at residue tyrosine 397 positioned at the juncture between the N-terminal and catalytic domains, which creates a high affinity binding site for the respective SH2 domains of each protein (Cobb et al., 1994; Schaller et al., 1994; Eide et al., 1995). Indeed tyrosine 397 is the major site of tyrosine phosphorylation of pp125Fak both in vitro and in vivo. The inability to autophosphorylate at tyrosine 397 does not impair the kinase activity of the enzyme in vivo, but abolishes the interaction with src. It appears that localisation of pp125Fak to the membrane is required for activation as a transmembrane anchored
chimaeric pp125Fak is constitutively active and tyrosine phosphorylated (Chan et al., 1994). The phosphorylation of pp125Fak at this position is a true autophosphorylation as kinase null pp125Fak mutants only become autophosphorylated at this position when co-expressed with active pp125Fak, and are not phosphorylated by other endogenous kinases (Chan et al., 1994). Upon cell adhesion to ECM, pp125Fak also becomes tyrosine phosphorylated on residues tyrosines 407, 576 and 577, which appear to be sites for phosphorylation by src family members, resulting in maximal kinase activity in pp125Fak (Calalb et al., 1995). Autophosphorylation of pp125Fak appears to regulate its interaction with src family members, possibly locking the src kinases in an activated state. Phosphorylation of paxillin by pp125Fak enables the adapter molecule crk to bind to paxillin, raising the possibility that pp125Fak phosphorylation events serve to recruit proteins to specific subcellular locations.

**Cellular targeting and protein-protein interactions of pp125Fak**

A number of domains within pp125Fak have been defined. Efficient localisation of pp125Fak to focal adhesions was initially thought to require sequences in the C-terminal domain as FRNK also localised efficiently to focal adhesions (Schaller et al., 1993). This focal adhesion targeting (FAT) sequence was narrowed down to a contiguous stretch of 159 amino acids within the C-terminus of the protein (Hildebrand et al., 1993) and more recently has been found to lie between residues 904-1040 (Hildebrand et al., 1995). pp125Fak forms a physical association in vivo with paxillin, the paxillin-binding site lying in the last 148 residues overlapping but distinct from the FAT sequence (Hildebrand et al., 1995). This interaction appears constitutive as it can be detected in cells taken into suspension. Talin has also been shown to interact with a 48 amino acid sequence within the C-terminus (residues 965-1012) of pp125Fak, independent of pp125Fak kinase activity and phosphorylation. The ability of integrin-mediated cell adhesion to stimulate pp125Fak tyrosine phosphorylation has also been shown to depend upon 13 amino acids within this putative talin binding site (Chen et al., 1995), leading the authors to hypothesise that talin is an upstream mediator of pp125Fak activation. In this regard pp125Fak has also been shown to interact directly with peptides mimicking the cytoplasmic tails of certain β-integrins, interacting via its N-terminal domain (Schaller et al., 1995), leading to an alternative hypothesis that clustering of integrins enables pp125Fak to autophosphorylate.

pp125Fak also interacts with a number of classical signalling proteins. When NIH3T3 cells spread on ECM components, the SH2/SH3 domain-containing adapter protein GRB2 associates in a signalling complex with pp125Fak. Tyrosine phosphorylation of pp125Fak at residue 925 creates a binding site for the SH2 domains of GRB2, and could possibly link adhesions with the Ras/MAP kinase signal transduction pathway. Indeed pp125Fak could be detected in immunoprecipitation of SOS, the adaptor protein which links GRB2 with the MAP kinase cascade through an interaction with the GRB2 SH3 domains (Schlaepfer et
The same interaction between pp125Fak and GRB2 has been detected upon stimulation of monocytes with macrophage colony stimulating factor. This also promoted interaction of pp125Fak/GRB2 complex with the GTPase dynamin (Kharbanda et al., 1995). In platelets, thrombin activation induces the translocation of phosphoinositide 3-kinase to the cytoskeleton. This translocation has been shown to involve specific interaction of the p85α subunit with both actin filaments and focal adhesion kinase. p85 was shown to interact with a proline-rich sequence within human pp125FAK (residues 706-711), via its SH3 domain, again possibly facilitating the localisation and activation of PI 3-kinase within specific subcellular locations. Indeed, association with pp125Fak increased the activity of PI 3-kinase (Guinebault et al., 1995).

The function of pp125Fak?
The correlation between the formation of focal adhesions and stress fibres, and the tyrosine phosphorylation of pp125Fak has implied that it is a kinase regulating cell adhesion. This may not however be the case. When cultured mouse aortic smooth muscle cells spread on fibronectin, pp125Fak is neither tyrosine phosphorylated nor exhibits tyrosine kinase activity, although stress fibres and focal adhesions form normally (Wilson et al., 1995). Moreover when platelets are activated by various factors to stimulate adhesion the activation of pp125Fak is a later event temporally distinct from the adhesive response (Lipfert et al., 1992).

More direct evidence for the true function of pp125Fak has come from analysis of mesodermal cells isolated from pp125Fak null mouse embryos. The pp125Fak knockout was lethal with development retarded and a general defect in mesoderm development (Llic et al., 1995). The mesodermal cells isolated from these embryos exhibited substantially reduced rates of migration compared to pp125Fak positive cells. The cells were still able to spread on fibronectin, but not as well as pp125Fak positive cells. Examination of the cytoskeleton revealed that rather than the normal stress fibres formed in the pp125Fak positive cells, the null cells possessed actin fibres around the cells periphery rather than the central region, and had numerous actin microspikes around the cell periphery. The cortical organisation of cortactin was diffuse signifying a disorganised cortical cytoskeleton. Although vinculin, talin and β1-integrins localised to focal adhesions, there were more smaller adhesions over the ventral surface of the cell, rather than having the peripheral distribution seen in normal cells. The results obtained in this study suggest that rather than controlling the formation of focal adhesions, pp125Fak regulates the turnover of focal adhesions associated with cell migration.

Interestingly, increased expression of pp125Fak has recently been shown to be correlate with an increase in invasive potential of metastatic colon and breast carcinomas and in high level sarcomas (Owens et al., 1995). It has been previously been shown to be present in
Figure 1.7. Domain structure of pp125Fak and p41 FRNK.
the majority of invasive cancers studied, and in all metastatic tumours (Weiner et al., 1993), and appears to be a marker associated with tumour invasiveness. pp125Fak may also regulate or facilitate the recruitment of various regulatory proteins to macromolecular signalling complexes specifically assembled at sites of adhesion, integrating signals from the extracellular matrix. Despite clear evidence from the pp125Fak null cells that it is not essential for adhesion formation, the true function of pp125Fak is still unclear.

1.5.2 Src family tyrosine kinases and the role of csk
V-src was one of the first tyrosine kinases discovered. It was identified as the oncogenic product of Rous sarcoma virus. The src family now consists of many enzymes of approximately 60 kDa, some identified as the products of oncogenes; yes, fgr, and lck, and others by homology screening fyn, yrk, hck, lyn, and blk (Erpel and Courtneidge, 1995). Most of these kinases show restricted tissue distribution with expression in specific haematopoietic cells. Only src, fyn and yes, and perhaps yrk are widely expressed. The domain structures and regulatory mechanisms are conserved throughout the family with only the first 60-80 residues of the total length of 500-530 residues being divergent between individual kinases. The conserved sequences can be divided into five domains. At the extreme N-terminus there is an N-terminal myristoylation required for localisation to the membrane via modification with a lipid moiety. Each member possess an SH3 and SH2 domain required for functional regulation of the protein, a kinase domain and a C-terminal non-catalytic tail (Cooper and Howell, 1993). The domain structure representative of src family members is shown in Fig. 1.8.

Regulation of src kinases by csk
The catalytic activity of src is controlled by the csk tyrosine kinase (C-terminal src kinase), which specifically phosphorylates tyrosine 527 (in chicken c-src), a mechanism that appears to regulate all members of the family. Tyrosine phosphorylation by csk creates a high affinity binding site for the SH2 domain of src which binds intramolecularly to its own C-terminal tail, the interaction being stabilised via the SH3 domain (Superti-Furga et al., 1993; Erpel et al., 1995; Superti-Furga and Courtneidge, 1995). Head-tail association prevents the molecule making any intermolecular interactions and precludes the kinase domain. This csk regulated control of src family members src, fyn and lyn is extremely important as csk knockout mice show a lethal embryonic phenotype, and constitutive activation of src kinases (Imamoto and Soriano, 1993; Nada et al., 1993). Analysis of embryonic fibroblasts isolated from these csk null mice identified two proteins, cortactin and paxillin, with increased tyrosine phosphorylation in response to the activated src family kinases. Other proteins of molecular weights 125, 95 and 58 also showed increased tyrosine phosphorylation, though their identities are unknown. The cells themselves exhibited a partially transformed phenotype and impaired cytoskeletal organisation (Nada et al., 1994). Overexpression of csk in HeLa cells results in localisation of the tyrosine
kinase to focal adhesions. This localisation was dependent on the SH2 and SH3 domains of csk rather than its catalytic activity, probably through forming a complex with paxillin and possibly pp125Fak. Csk overexpression also caused the cells to become less well spread and the vitronectin receptor was found to redistribute into podosome like structures (Bergman et al., 1995).

Upon activation of src, tyrosine 527 often becomes dephosphorylated with a concomitant phosphorylation occurring at tyrosine 416 within the kinase domain (Cooper and Flowell, 1993). Mutations within the catalytic domain, SH2, SH3 and C-terminal regions have all been shown to activate src. Relief of the csk-mediated downregulation of src and hence activation could occur in a number of ways. Firstly through dephosphorylation of tyrosine 527. Activation of CD45, a transmembrane tyrosine phosphatase which mediates signalling through the T-cell receptor, results in activation of src family kinases lck and fyn. Moreover CD45 has been shown to directly act on lck resulting in phosphorylation of the T-cell receptor and recruitment of the tyrosine kinases Zap70/syk to a signalling complex (Cahir-McFarland et al., 1993; Weiss and Littman, 1994). Transfection of fibroblasts with protein tyrosine phosphatase α results in constitutive activation of c-src and transformation of the cell (Zheng et al., 1992). Indeed a direct association has been detected between src and a protein tyrosine phosphatase PTPD1, one of the ERM family of proteins, which is itself a substrate for src (Moller et al., 1994). Secondly, competition between different src SH2 binding sites for the SH2 domain of src may occur. This is the method by which tyrosine kinase receptors activate src. Interestingly the SH2 domain of src and fyn can associate with tyrosine phosphorylated residue 397 of pp125Fak possibly providing a mechanism by which src can be recruited and activated at sites of adhesion (Cobb et al., 1994; Schaller et al., 1994; Eide et al., 1995). Finally a similar competition for the SH3 domain may occur. Although no evidence for this has been found, src interacts with paxillin via the src SH3 domain (Weng et al., 1993).

**Cellular effects of src-mediated signalling**

Src family members are involved in mediating a wide range of cellular signals. Members of this family of kinases are required for the mitogenic response to growth factors in fibroblasts and the activation of platelets and lymphocytes. By microinjection of dominant negative mutants it has been shown that src and fyn are required for a response to PDGF, and indeed interact with a number of tyrosine kinase and non-tyrosine kinase transmembrane receptors. Functional redundancy occurs within the src family as inhibition of both kinases was required to inhibit the PDGF response (Twamley-Stein et al., 1993). Src family members are also required for signalling via the CSF-1 receptor and EGF receptor.

Early work on v-src revealed that transformation of cells with RSV resulted in tyrosine
phosphorylation of numerous cellular proteins and redistribution of peripheral focal adhesions and stress fibres to podosome-point adhesions distributed all over the ventral surface of the cell (Rohrschneider, 1980). Increased tyrosine phosphorylation of a number of cytoskeletal proteins such as vinculin, talin, ppl25Fak, paxillin, cortactin, tensin are all associated with RSV transformation (Glenney and Zokas, 1989). Targeting of v-src to adhesion plaques is sufficient to transform CEF (Liebl and Martin, 1992). V-src has also been shown to perturb N-cadherin mediated cell-cell adhesion in CEF, by disrupting the interaction with catenins (Hamaguchi et al., 1993). The proto-oncogenic forms of c-yes and c-src have been shown to be normally associated with cell-cell junctions (Tsukita et al., 1991). Src family members must play a role central to regulation of adhesion, as perturbation of the tight control exerted on members of this family has such profound effects on cellular adhesion processes.

The role that src family members normally play in adhesion regulation is poorly understood. Upon platelet activation c-src becomes associated with the insoluble cytoskeletal fraction, in a manner that requires both aggregation and actin polymerisation (Oda et al., 1993; Horvath et al., 1992). The interaction has been shown to be mediated by the SH2 domain and to depend on residues within the catalytic domains of src (Okamura and Resh, 1994). In PCI2 cells src is required for neurite outgrowth in response to nerve growth factor and fibroblast growth factor (Kremmer et al., 1991). In fibroblasts src and fyn are found associated with a number of proteins associated with adhesions, not only ppl25Fak and paxillin, but also a 110 kDa protein actin filament associated protein (AFAP), which is also a substrate of src, in a manner that requires both the SH2 and SH3 domains of src (Flynn et al., 1993). Moreover tyrosine kinase inhibitors that preferentially inhibit src family tyrosine kinases interfere with adhesion associated signalling events. Although the evidence strongly supports a role for src family members in adhesion regulation and signalling, the specific role of the protein is as yet unknown.

Numerous single knockouts of src family members in mice have demonstrated that specific members are important to particular cell types. Though no mouse lacking one member of the src family is an embryonic lethal (Erpel and Courtneidge, 1995), progress has been made with the knockout of both src and fyn in mice with a csk negative background (Thomas et al., 1995). Csk knockouts are embryonic lethal. However fibroblasts can be isolated from embryos with disruptions in one or both src and fyn genes, prior to death at 9 or 10 days gestation. Disruption of src and fyn removes the kinases from a genetic background in which they are constitutively active, but not transforming. This reveals which of the src family tyrosine kinases are responsible for the cellular changes associated with the csk phenotype. This approach has revealed that hyperphosphorylation of cortactin and tensin is dependent on src, whereas ppl25Fak and paxillin is partly dependent upon fyn. Moreover examination of the phenotype of fibroblasts isolated from src/cks− embryos
Figure 1.8. Domain structure of the src family members pp60src and pp59fyn.
Figure 1.9. A model for integrin mediated signalling. (Modified from Schaller and Parsons, 1994). A schematic summary coordinating aspects of Sections 1.4 and 1.5. A very simplified model showing one possible way in which certain focal adhesion proteins, pp125Fak, paxillin, src, csk and crk may interact to regulate signal transduction from sites of adhesion. This model incorporates some of the interactions between the "signalling" proteins discussed in previous pages. Engagement of integrins leads to recruitment of pp125Fak to the cytoplasmic tail of β1-integrin. Once recruited to the membrane/site of adhesions pp125Fak autophosphorylates and interacts with c-src/fyn and paxillin. This complex of tyrosine phosphorylated proteins and tyrosine kinases creates the perfect environment for the recruitment of SH2 domain containing proteins such as crk which binds the nucleotide exchange factor C3G creating a possible link with small GTP-binding proteins (Section 1.7). The link to the nucleus may be facilitated through the small GTP-binding proteins. Src/fyn activity is regulated by the csk which binds to paxillin and pp125Fak. A number of important proteins are omitted from this model including tensin, pp130cas and pp120AFAP.
and fyn''/csk'' embryos has implied that within the csk null background, the podosome-like
distribution of cortactin and actin observed is a result of src action. Removal of src
restores actin stress fibres and the normal distribution of cortactin. Knockout of fyn did
not appear to affect cell morphology. From the data obtained, the authors conclude that
csk regulated src mediates an effect on stress fibre organisation through tyrosine
phosphorylation of cortactin. Both src and fyn effect the organisation of adhesions via
tyrosine phosphorylation of tensin, paxillin and pp125Fak. Although this approach has
indicated putative targets of these two kinases in the cell, deduction of the positive effects
of each kinase is not possible. Perhaps more positive information could be obtained from a
multiple src family knockout, and the restoration of the individual proteins within those
genetic backgrounds.

1.5.3 The protein kinase C family
Structure and regulation
The name protein kinase C (PKC) describes a large family of serine/threonine kinases that
play fundamental roles in regulating numerous physiological processes. PKC was initially
characterised as an enzyme activated in vitro by Ca\(^{2+}\), phospholipid and diacylglycerol
(DAG). The PKC family now consists of more than 12 different polypeptides which have
varied tissue distribution and expression. The different isoforms can be divided into three
groups, the classical Ca\(^{2+}\) and DAG dependent cPKC, the novel Ca\(^{2+}\) independent nPKC
isoforms and atypical Ca\(^{2+}\) and DAG independent aPKC isoforms (see Table 1.3). The
three groups of PKCs share a number of conserved regions and their domain structure is
represented in Fig. 1.10. (Stabel and Parker, 1991; Clemens et al., 1992; Hug and Sarre,
1993). In addition to the PKC isoforms described here, a number of protein kinase C-
related gene products have been identified which are thought to describe a novel family of
kinases, the protein kinase C related kinase (PRK 1-3) family (Palmer et al., 1994).

Classical PKC isoforms are activated as a result of hydrolysis of PIP2 by PLC\(_{\gamma}\) or \(\beta\) to
produce IP3 and DAG. DAG binds directly to the phorbol ester binding site in PKC, while
IP3 releases Ca\(^{2+}\) from intracellular stores. Novel PKCs can be activated by DAG alone,
though more recently it has become obvious that a number of acidic phospholipid
metabolites are capable of activating different PKC isoforms (Liscovitch and Cantley,
1994). Interestingly PIP2 and PIP3, two lipid second messengers associated with
regulating actin polymerisation and the activity of various cytoskeletal proteins, have been
shown to be capable of activating a number of novel isoforms of PKC, \(\delta\), \(\varepsilon\) and \(\eta\), in vivo
(Toker et al., 1994). Atypical PKCs seem to be activated by a number novel lipid products
(Dekker and Parker, 1994). PKCs are negatively regulated by an intramolecular
interaction. They contain a regulatory domain consisting of the pseudosubstrate and
phorbol ester binding site. In the absence of positive stimuli the pseudosubstrate site
interacts with the kinase domain blocking the catalytic domain by interacting with the
**Table 1.3. The PKC family of Serine/Threonine kinases.** The PKC family is divided into three main groups classical, novel and atypical. Where information is available the molecular weight and expression of the different isoforms are indicated.

<table>
<thead>
<tr>
<th>Classical PKCs</th>
<th>α (80 - 81 kDa) - widespread expression</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ca²⁺ and DAG dependent.</td>
<td>β1 (79 - 80 kDa) - wide range expression</td>
</tr>
<tr>
<td></td>
<td>β2 (80 kDa) - wide range expression</td>
</tr>
<tr>
<td></td>
<td>γ (77 - 84 kDa) - central nervous system</td>
</tr>
<tr>
<td>Novel PKCs</td>
<td>δ (74 - 79 kDa) – widespread expression</td>
</tr>
<tr>
<td>Ca²⁺ independent and DAG dependent.</td>
<td>ε (89 - 96 kDa) - widespread expression</td>
</tr>
<tr>
<td></td>
<td>η (78 - 80 kDa) - very restricted expression</td>
</tr>
<tr>
<td></td>
<td>θ (79 kDa) - very restricted expression, skeletal muscle</td>
</tr>
<tr>
<td></td>
<td>μ - no data available</td>
</tr>
<tr>
<td>Atypical PKCs</td>
<td>ζ (76 - 80 kDa) - widespread expression</td>
</tr>
<tr>
<td>not affected by DAG or Ca²⁺.</td>
<td>λ - very restricted expression</td>
</tr>
<tr>
<td></td>
<td>i - no data available</td>
</tr>
</tbody>
</table>
Figure 1.10. Representation of the domain structures of the different PKC isoforms. Alpha, beta I and II and gamma represent the classical PKC isoforms, delta and epsilon the novel PKC isoforms, and zeta and eta the atypical PKC isoforms. The proteins are divided into two domains the N-terminal regulatory domain and the C-terminal catalytic domain. The C1 region contains the pseudosubstrate and one or two zinc fingers (blue). The C2 region is present only in the classical PKCs and confers the calcium sensitivity on these proteins. The conserved kinase domains consisted of regions C3 and C4, with C3 containing the ATP binding site.
substrate pocket and maintaining the enzyme in the inactive form. The pseudosubstrate site is a short polypeptide that contains the preferred consensus sequence for phosphorylation by that isoform, but with the serine replaced with a non-phosphorylatable residue such as alanine (Stabel and Parker, 1991; Dekker and Parker, 1994). Binding of cofactor (such as DAG) to the protein relieves this interaction enough to allow PKC to phosphorylate its substrate. A second element essential for full PKC activation is autophosphorylation on either a serine or a threonine residue. For example phosphorylation of PKCα on threonine 497 is a preliminary event required for full activation of the protein (Cazaubon et al., 1994). Phosphorylation by other kinases can also serve to potentiate the activation of specific protein kinase C isoforms. Tyrosine phosphorylation of PKCβ by either src family or receptor tyrosine kinases is associated with activation in vitro (Li et al., 1994a), and is activated in vivo by treatment of cells with PDGF (Li et al., 1994b). Once activated, PKC isoforms exhibit rapid redistribution in cellular location, usually to become associated with the membrane or particulate fractions of the cell (Dekker and Parker, 1994). Once activated and associated with the membrane, PKC can be inactivated by cleavage of the protein by calpain II to yield the N-terminal regulatory domain and the C-terminal catalytic domain (Croall and Demartino, 1991). Long term stimulation of PKC ultimately results in downregulation of the expression of the protein.

**Protein interactions by PKC**

PKC has been shown to interact with a number of cellular proteins, some of which are also substrates. A variety of isoforms, α, β, γ, ε, and ζ interact with the pleckstrin homology (PH) domain of the bruton tyrosine kinase (Btk) (Yao et al., 1994). PH domains are novel domains found in a number of proteins thought to play a role in mediating protein/protein interactions, and may also facilitate lipid binding (Gibson et al., 1994). PKC appears to negatively regulate Btk activity by serine phosphorylation occurring as a result of this interaction both in vitro and in vivo (Yao et al., 1994). By chemical crosslinking PKCα has been shown to interact with vinculin in vitro in a Ca²⁺ and phospholipid dependent manner, and has also been isolated from REF52 fibroblasts complexed with annexins (Hyatt et al., 1994). Analysis of PKCα indicates that it appears to interact with phospholipid-binding proteins in a manner that requires the pseudosubstrate domain (Liao et al., 1994a). This raises the possibility that PKC could target substrates by interacting with the lipids bound to respective target proteins. Consistent with this is the observation that PKC interacts with two proteins (110 kDa and 115 kDa), in cell lysates immobilised on nitrocellulose, in a manner dependent on phosphatidylinerine but not other phospholipids. Unfortunately, the identity of these protein is unknown, although interestingly, they were enriched in cytoskeletal fractions (Wolf and Sahyoun, 1986). Finally two intracellular receptors have been identified for PKC, termed RACKs (receptors for activated C-kinase), which have molecular weights of 33 and 30 kDa. These proteins
interact with activated PKC and possibly play a role in localising the proteins to the membrane/particulate fraction. The interaction with RACKs is thought to depend on phosphatidylinerine, Ca²⁺ and DAG, as well as a RACK-binding site located in the centre of PKC (Mochley-Rosen et al., 1991a; Mochley-Rosen et al., 1991b). A cDNA encoding a 36 kDa PKC-binding protein RACK1 has been cloned which shows homology to the β subunit of G proteins (Ron et al., 1994).

**Cellular effects of PKC**

Despite the identification of large numbers of PKC isoforms and PKC substrates, little is known about the direct *in vivo* targets of the PKC isoforms in relation to signal transduction. PKC is generally activated *in vivo* using the phorbol ester TPA. TPA interacts with the phorbol ester-binding site mimicking the action of DAG. TPA has different effects on cells in a concentration dependent manner, with chronic prolonged treatment leading to downregulation of expression of certain PKC isoforms. Treatment of HL60 cells with different doses of TPA induces either proliferation at low concentrations, or differentiation into macrophages at high concentrations (Traynor and Clemens, 1992), perhaps because different concentrations of TPA activate different PKC isoforms, or because high doses of PKC induces rapid downregulation of certain PKC isoforms. PKC has been shown to play a role in a number of signalling pathways. p21Ras and PKC cooperate in transformation, overexpression of PKC conferring susceptibility to transformation by the H-ras oncogene (Hsiao et al., 1989). Evidence also suggests that PKC may function on the ras pathway, acting upstream of ras (Downward et al., 1990; Marquardt et al., 1994). Indeed treatment of cells with TPA also results in stimulation of MAP kinase activity (Adams and Parker, 1991; Nori et al., 1992) and the phosphorylation of components of the transcriptional and translational machinery (Boyle et al., 1991; Morley et al., 1991). In 3T3-L1 cells, PKC is required for the activation of the MAP kinase cascade by leukaemia inhibitory factor (LIF) (Schiemann and Nathanson, 1994).

TPA activates numerous PKC isoforms within the same cell, which limits the conclusions which can be drawn from its use. Another problem is that TPA can also bind to other cellular proteins such as n-chimaerin and une-13 (Areces et al., 1994; Kiley and Jaken, 1994). Although no functional consequences of such interactions have been demonstrated, it is possible that other cellular proteins are also affected by TPA. More recent efforts at defining specific roles of PKC in signal transduction have centered around the overexpression and inhibition of specific isoforms in model cell systems. Overexpression has revealed for example that PKCα and δ are involved in differentiation of mouse myeloid cells (Dekker and Parker, 1994). Expression of PKCδ was elevated in SV40 transformed cells that grow in soft agar, with the anchorage dependent growth inhibited by overexpression of the regulatory domain of PKCδ. Interestingly overexpression of the regulatory domain of PKCα enhanced the ability to grow in soft agar (Liao et al., 1994b).
Dominant negative mutants of PKC have also been created by mutating the ATP binding site. This approach has shown that PKC\(\gamma\) is required for oocyte maturation in *Xenopus* (Deldcer and Parker, 1994). Such approaches coupled with antisense and gene knockout may provide more conclusive information concerning the functions of the PKC isoforms, particularly the redundancy that may exist within the family. Published evidence indicates that PKC isoforms are involved in processes that regulate both growth and differentiation.

**PKC as a regulator of cell adhesion**

Despite the limitations associated with the use of TPA to study PKC-mediated signalling, much of the data supporting an involvement of PKC in adhesion has come from use of this reagent. PKC was first thought to play a role in adhesion regulation as phorbol ester treatment of CHO cells increased cell spreading on fibronectin, even in the presence of anti-functional integrin antibodies and peptides corresponding to the cell-binding motif (Danilov and Juliano, 1989). REF were shown to be unable to spread on the central cell-binding domain of fibronectin unless cells were treated with TPA. Moreover the differential inhibitory effects of a combination of kinase inhibitors, H7 and HA1004, on REF cell spreading on fibronectin indicated an involvement of PKC (Woods and Couchman, 1992). More recently, specific inhibitors of PKC have become available. The PKC specific inhibitor calphostin C inhibits CHO cell spreading on fibronectin, the PKC activation being a pre-receptor activation event, i.e PKC activity is required for receptor to engage ligand (Vuori and Ruoslahti, 1993). A similar situation has been observed in lymphocytes, where TPA treatment induces LFA-1-dependent aggregation by activation of the receptor (Tominaga et al., 1993). Moreover treatment of CHO cells with TPA also stimulated a more spread phenotype, and interestingly appeared to potentiate the tyrosine phosphorylation of pp125Fak (Vuori and Ruoslahti, 1993). PKC has also been reported to play a positive role in E-cadherin mediated formation of adherens junctions (Lewis et al., 1995). The above evidence indicates a positive role for TPA responsive PKCs in regulating adhesion. Confusingly, when serum-starved Swiss 3T3 cells are stimulated with LPA in the presence of TPA, no formation of stress fibres and adhesions is seen (Ridley and Hall, 1994), implying that PKC has a negative role in this situation.

One PKC isoform, PKC\(\alpha\), has been localised to focal adhesions in one cell line REF52 rat embryo fibroblasts, but redistributes to a nuclear location upon TPA treatment (Jaken et al., 1989). In normal REF52 cells PKC\(\alpha\) is also tightly associated with the cytoskeleton, but in SV40 transformed cells it is no longer localised to adhesions or the cytoskeleton, though in both situations it associates with the particulate membrane fraction (Hyatt et al., 1990). This localisation has not presently been confirmed in any other cell line or using another PKC\(\alpha\) antibody. At the protein level, two cytoskeletal proteins vinculin (Werth et al., 1983; Werth and Pastan, 1984) and talin (Lichfield and Ball, 1990a; Lichfield and Ball, 1990b; Bertagnolli et al., 1993) have been reported to be substrates for PKC *in vitro* and *in vivo*.
vivo, while a number of other proteins such as pp125Fak (Hatai et al., 1994) and paxillin (Turner and Miller, 1994) are reported to be serine/threonine phosphorylated and contain consensus phosphorylation sites for PKC. PKC would appear to have a role in regulating cell adhesion though identification of precise roles has proved elusive.

1.6 Regulation of tyrosine kinase action, protein tyrosine phosphatases

1.6.1 Signalling events regulated by protein tyrosine phosphatases.

Although much consideration has been given to the role that proteins tyrosine kinases play in regulating cell adhesion and associated signalling processes, little effort has been directed to defining the role of protein tyrosine phosphatases (PTPases). PTPases play fundamental roles in regulating cell growth (Fischer et al., 1991; Schlessinger and Ullrich, 1992), the cell cycle (Gautier et al., 1991; Gyuris et al., 1993) and cytoskeletal integrity (Bennett et al., 1993) in response to a variety of external stimuli. Over forty PTPases have been described to date, which can be divided into three basic subtypes. The intracellular phosphatases consist of a central phosphatase domain flanked by N- and C-terminal auxiliary regions which usually possess regulatory and targeting domains. The transmembrane receptor-like PTPases consist of an extracellular domain of varied composition, a transmembrane domain and either one or two cytoplasmic phosphatase domains. The third class are the dual specificity PTPases which utilise phosphoserine and phosphothreonine as substrates as well as phosphotyrosine (Stone and Dixon, 1994).

By virtue of the fact that they reverse the action of tyrosine kinases, PTPases are generally regarded as playing negative roles in signal transduction. However situations have been described where PTPase are positive regulatory elements of signalling pathways. The action of PTPases has been most studied in cells of erythroid and haematopoietic lineage. Two PTPases have been shown to play positive roles in signal transduction. The transmembrane PTPase CD45 is essential for signalling associated with lymphocyte activation following stimulation of antigen receptors (Tonks et al., 1988). Stimulation of the antigen receptor on a CD45-deficient cell line failed to elicit cellular activation (Pingel and Thomas, 1989). More specifically CD45 controls the activation of LFA-1 allowing an interaction with ICAM-1 or ICAM-3, leading to cell aggregation. The CD45 signalling phenomenon has been extended to include activation of B cells and natural killer cells (Brady-Kalnay and Tonks, 1995). Upon activation of T-cells, CD45 dephosphorylates the C-terminal tyrosine of the src family member p56 lck (lck) (equivalent to tyrosine 527 in src) relieving its negative regulation and so activating the kinase activity (Mustelin et al., 1989). The cytosolic PTPase syp (otherwise known as PTP1D, SHPTP and PTP2C) also has a positive signalling function. It possesses two SH2 domains and appears to act as an adapter molecule coupling tyrosine kinase growth factor receptor to their downstream targets, promoting the interaction of GRB2 and sos (Li et al., 1994c). Phosphatases do of course have negative regulatory roles. The best example is that of PTP1C (also known as
HCP, SHP and SHPTP1), which terminates proliferative signals resulting from stimulation of the erythropoietin (EPO) receptor (Klingmuller et al., 1995). The EPO receptor lacks intrinsic tyrosine kinase activity which is supplied via interaction with a non-receptor tyrosine kinase, JAK2. Activation of EPO-receptor induces tyrosine phosphorylation and activation of JAK2, and phosphorylation of tyrosine 429 in the EPO receptor, creating a high affinity binding site for the SH2 domains of PTP1C, which dephosphorylates and inactivates JAK2 (Imboden and Koretsky, 1995). PTP1C itself is also tyrosine phosphorylated by Ick on residue 536 and 564 (Lorenz et al., 1994), though the significance of this is unclear. The recruitment of the phosphatase not only terminates the signal, but the mechanism of recruitment controls the period for which signal is transduced. PTP1C also appears important in controlling signalling in haematopoietic cell lines. It is known to associate with the receptor tyrosine kinase c-Kit and the CSF-1 and IL-3 receptors, while mutation of the PTP1C gene causes developmental abnormalities in all haematopoietic cell lines (Schultz et al., 1993; Yi and Ihle, 1993).

1.6.2 The transmembrane PTPases
The extracellular domains of many of the transmembrane PTPases are homologous to cell adhesion molecules. These domains are repeat regions homologous to the fibronectin type III repeat which contains the RGD-motif in fibronectin, and the Ig repeats of N-CAM (Brady-Kalnay and Tonks, 1995). Due to these regions of homology, such transmembrane PTPases are thought to be possible adhesion receptors. Indeed two receptor PTPases, PTP ι and PTP κ have been shown to be capable of mediating cell-cell aggregation through a homophilic interaction, the receptor binding to an identical molecule on an opposing cell (Brady-Kalnay et al., 1993; Gebbink et al., 1993; Sap et al., 1994). This binding appears to be mediated by the Ig domains, with the specificity of the interaction determined by the MAM domain, an extracellular motif contained in both phosphatases (Beckman and Bork, 1993). Such PTPases are thought to play a role in regulating cell-cell junctions. PTP ι has also has a region of homology with the cadherin intracellular domain and has been shown to localise to complexes containing α- and β-catenin, and binds directly to the E-cadherin (Brady-Kalnay et al., 1995). PTP κ also contains this region of cadherin homology, leading to the suggestion that perhaps tyrosine phosphorylation at sites of cell-cell adhesions is controlled by such PTPases.

PTPases in the regulation of adhesion
The role that PTPases play in regulating cell-matrix adhesion is poorly understood. Treatment of CEFs with trypsin or PBS leads to a rapid decrease in content of tyrosine phosphorylated cellular proteins. This is due to the activation of a tyrosine phosphatase(s) which can be inhibited by the tyrosine phosphatase inhibitors, sodium orthovanadate (vanadate) and phenyl arsine oxide (PAO) (Maher, 1993). Recently, a more detailed study has employed these same tyrosine phosphatase inhibitors. Treatment of CHO cells with
both inhibitors modulates tyrosine phosphorylation of cellular proteins including pp125Fak. The PTPase activity responsible for the dephosphorylation of pp125Fak and other cellular protein appears to be activated upon disruption of the cytoskeleton, as cytochalasin D treatment of cells led to dephosphorylation of pp125Fak. Moreover the cytochalasin D induced disruption of the cytoskeleton and focal adhesion was prevented by PAO treatment (Defilippi et al., 1995). Further evidence for the involvement of PTPases in adhesion regulation is provided by the observation that vanadate promotes the spreading of neutrophils on ECM substratum (Bennett et al., 1993). Activation of platelets also results in the regulation of PTPases through cytoskeletal reorganisation and tyrosine phosphorylation. Two PTPases PTP1B and PTP1C become associated with the cytoskeleton upon platelet activation (Ezumi et al., 1995). However by inference from their function in other cell types, it is likely that these PTPase are involved in signal termination rather than regulation of the cytoskeleton.

A number of PTPases could play a role in regulating cell-matrix adhesion, and more specifically the formation of focal adhesions. The most obvious candidate is the transmembrane receptor PTPase the LAR phosphatase, one of a family of PTPases with Ig and FN-type III domains. LAR has recently been shown to localise to focal adhesions, a localisation mediated by a LAR-associated protein LIP1 (LAR interacting protein) (Serra-Pages et al., 1995). It is reasonable to speculate that in certain cell types, LAR plays a role in regulating focal adhesion disassembly but as it is not widely expressed, other PTPase must be involved. The ERM family of proteins also contains four members which are PTPases, PTPD1, its close relation PTPD2, PTPH1, PTPMEG and PTPL1 (Gu et al., 1991; Yang and Tonks, 1991; Moller et al., 1994; Saras et al., 1994). These proteins are capable of localising to the membrane through the region of homology with ezrin radixin and moesin (Algrain et al., 1993), and as such they may well be localised to the correct subcellular location, although this remains to be established.

1.7 The role of small GTP binding proteins
1.7.1 Ras superfamily members - ras and the rho subfamily members.

The ras superfamily of low molecular weight (small, c. 21 kDa) GTP-binding proteins act as molecular switches in a wide range of biological processes. As many as 50 members of this superfamily have been described to date which are divided, on the basis of sequence homology, into five subfamilies: ras, rab, arf, ran and rho. Rab and arf family members are involved in regulation of vesicular transport, while the ran family members regulate nuclear import (Vojtek and Cooper, 1995). In this section, the other two subfamilies, ras and rho will be discussed. Ras is the prototypic small GTP-binding protein, with the ras family members (H-ras, K-ras, R-ras, TC21, rap1A/rap1B, and rap2A/rap2B) shown to control processes associated with cell growth and differentiation (Vojtek and Cooper, 1995). The rho family members (edc42/G25K, rac1, rac2, rhoA, rhoB and rhoC) have
been shown to play important roles in the regulation of the actin cytoskeleton (Ridley, 1994; Takai et al., 1995; Nobes and Hall, 1995b). A general representation of the ras/rho like GTP-binding proteins is shown in Fig. 1.11.

These proteins time the transmission of cellular signals by hydrolysing GTP. They are active when in the GTP-bound form and transduce signals to downstream effector proteins. In the GDP-bound form the proteins are inactive, and incapable of transducing a signal. The cycling between the GTP-bound active form and the GDP inactive form is controlled by a variety of positive and negative elements which interact directly with the GTP-binding protein. The GTP bound form is promoted by guanine nucleotide dissociation stimulators (GDS), otherwise known as guanine nucleotide exchange factors (GEF), which catalyse the dissociation of GDP. As the ratio of GTP to GDP in the cell is extremely high it is most likely that the GDP will be replaced with GTP, resulting in an active conformation. The protein is negatively regulated by two factors. The intrinsic rate of GTP hydrolysis by small GTP-binding proteins is low. This rate of hydrolysis is stimulated by the GTPase activating proteins (GAPs), such that when associated with a GAP, the protein is predominantly in the GDP bound form. A second negative element thought to control the activity of the proteins is the guanine nucleotide dissociation inhibitor (GDI), which is confusingly also classed as a guanine nucleotide exchange factor (GEF). These factors inhibit the dissociation of the nucleotide from the GDP bound form keeping the protein in the inactive state (Takai et al., 1995; Vojtek and Cooper, 1995).

For the purposes of simplicity, the signalling events mediated by the small GTP-binding proteins ras, rho, rac and cdc42 will divided into three sections; ras; rho and rac/cdc42.

1.7.2 The ras-mediated signal transduction pathway.
Ras is central to many growth factor/ mitogen mediated signalling pathways that stimulate DNA synthesis and gene expression. The ras gene is one of the most frequently mutated genes in human cancer. Mutation at residues glycine 12 and 13, glutamine 61 have been identified as conferring oncogenic properties on proteins isolated from tumours (Bos, 1988). The mutation of glycine 12 to valine 12 has proven invaluable in studying ras and other GTP-binding proteins as this constitutively activates the protein by inactivating the GTPase, so maintaining it in the active GTP bound form. The effector domain of p21ras has been shown, by deletion analysis, to lie between residues 32-40 which also is the region of interaction with rasGAP. The GTPase function of the enzyme requires residues in a number of domains, residues 10-17, 53-62, 112-119 and 146 (Hall, 1990).

Ras serves to link cell surface receptors receiving mitogenic signals from the extracellular environment to the cytoplasmic kinase cascades that ultimately regulate events within the nucleus. The same pathway is found in a wide variety of organisms ranging from
General domain structure of the small GTP-binding proteins.

Figure 1.11. Representation of the general domain structure of the ras-like small GTP-binding proteins. The residues required for GTPase function (red) are taken from those defined for ras. Positions of activating mutations are marked with arrows, and are consistent for ras, rho, rac and cdc42, except for the position of the dominant negative mutation which does not occur in rho. Where the residues vary for rho the residue number is indicated. The effector domain is represented in green. The hypervariable region seen in ras and rho is represented in blue. The prenylation motif is represented in orange.
Drosophila and C. elegans to mammals. In mammalian cells, stimulation of a receptor tyrosine kinase such as the EGF receptor leads to autophosphorylation of the receptor. This in turn creates tyrosine phosphorylated SH2-binding motifs, leading to the recruitment of a number of signalling proteins, via their SH2 domains to form a signalling complex. One such protein recruited to the receptor is an adapter protein known as grb2 (sem5 in C. elegans and drk in Drosophila), which contains one SH2 domain and two SH3 domains. Via its SH3 domains, grb2 couples the receptor to a second protein sos (the mammalian homologue of the Drosophila protein son of sevenless, denoted msos for mouse and hsos for human). Sos is a guanine nucleotide exchange factor for ras and promotes the accumulation of membrane associated GTP-bound ras, generating downstream signals (Buday and Downward, 1993; Egan et al., 1993; Li et al., 1993; Rozakis-Adcock et al., 1993).

Ras and ras-related proteins (such as rho) are recruited to the membrane by virtue of post-translational isoprenylation modifications at the C-terminus. A signal sequence for prenylation, the CaaX motif is carried within the C terminus of the ras proteins (where C represents cysteine, a represents an aliphatic residue). Modification by farnesylation (addition of hydrophobic thioether linked prenyl groups) creates a hydrophobic tail which is required for interaction with the membrane (Marshall, 1993), and appears essential for effector function but not for interaction with GAPs. Ras cycles between the membrane and the cytosol with both interactions mediated by the prenyl modification. As well as mediating membrane interaction, this modification may also mediate protein-protein interaction with GDI by insertion into a hydrophobic pocket. This interaction relocates the GTP-binding protein from the membrane to the cytosol (Takai et al., 1995). Once recruited to the membrane and activated by sos, GTP-ras then acts to recruit the serine/threonine kinase raf to the membrane (Vojtek et al., 1993; Spaargaren et al., 1994), where raf becomes tyrosine phosphorylated and activated, probably by membrane bound src (Marais et al., 1995). Ral in turn signals to the so called MAP kinase cascade, a linked series of kinases. Ras activates raf, a MAPK kinase kinase (MAPKKK), which signals to a MAPKK called MEK, and finally to a MAP kinase, ERK (Vojtek and Cooper, 1995). A parallel MAP kinase cascade is activated by the rho-related proteins cdc42 and rac with both pathways converging at the serum response element (Whitmash et al., 1995). Ras may also play a direct role in regulating the production of lipid second messengers, as it has recently been shown that PI 3-kinase is a direct target of ras. This interaction occurs between the catalytic subunit of PI 3-kinase and the effector region of ras (Downward et al., 1990). Moreover COS cells transfected with ras, but not with raf, exhibited higher levels of 3'-phosphorylated phosphoinositides, while PC12 cells transfected with the ras dominant negative mutant failed to produce these lipids in response to growth factors (Rodriguez-Viciana et al., 1994).
Although growth factor activation of the ras pathway is important in mitogenesis, it also has a profound effect on the cytoskeleton. Stimulation of cells with PDGF initially leads to a disruption of the actin cytoskeleton (Ridley et al., 1992), which could possibly occur through a ras associated protein rasGAP. RasGAP (a 120 kDa SH2/SH3-containing phosphoprotein), the negative regulator of ras interacts with two proteins p62 an RNA binding protein, and p190 which has GAP activity against rho (Settleman et al., 1992b; Ridley et al., 1993). The C-terminus of rasGAP stimulates the GTPase activity of ras (Marshall et al., 1989). The N-terminal region of the protein contains the SH2 and SH3 domains through which p62 and p190 rhoGAP interact with rasGAP (McGlade et al., 1993). Upon activation of the EGF-receptor, the majority of the cellular p190 (rhoGAP) is associated with rasGAP, and both become tyrosine phosphorylated (Ellis et al., 1990).

Expression of the N-terminal region of rasGAP in Rat2 cells leads to disruption of the cytoskeleton, decreased fibronectin binding, decreased cell adhesion and disruption of focal adhesions. The p190/rasGAP complex possess rhoGAP activity. Therefore the cellular effects of the N-terminal region of rasGAP may arise because p190 rhoGAP is promoting the GDP-bound inactive form of rho, causing disassembly of the cytoskeleton (McGlade et al., 1993). This interaction may provide a point of integration or cross-talk between the ras and rho/rac mediated signalling pathways.

1.7.3 Signalling mediated by the rho subfamily

The rho proteins show around 30% homology to the ras proteins and have the same general domain structure. There are three rho protein A, B, and C in mammals which show 80% identity, differing only in the C-terminal region (Ridley, 1994). They are expressed to varying degrees in a wide range of tissue types, within which they appear to be differentially regulated (Fritz et al., 1994). The effector domain of rho lies between residues 34-42, a region equivalent to the ras effector domain (Hall, 1990). Like ras, rho proteins are prenylated within their C-termini, possessing the unique carboxy terminal motif CaaL (a-representing an aliphatic residue), at which rho is geranylgeranylated on the cysteine, a modification also necessary for localization of rho to the membrane and hence activation and function (Takai et al., 1995). Rho is also constitutively activated by a mutation glycine 14 to valine 14, a property that has proved useful in studying rho (Garrett et al., 1989), though examination of human tumours reveals that rho does not appear to be a proto-oncogene (Moscow et al., 1994). A novel feature of the rho A, B and C proteins is that they can be inhibited by ADP ribosylation on asparagine 41 by the bacterial toxin C3 transferase (Aktories et al., 1989; Chardin et al., 1989; Fritz and Aktories, 1994). Importantly the action of C3 transferase has no effect on the intrinsic biochemical properties of the protein or its ability to interact with rhoGAP (Paterson et al., 1990). Rho has been shown to be modified by a second bacterial toxin, Bordetella bronchisaptaica derronecrotizing toxin. This toxin activates rho such that it overcomes C3 transferase inhibition, though the mechanism of action is unclear (Horiguchi et al., 1995). Like ras,
rho proteins cycle between the cytosol (inactive) and the membrane (active). By microinjection of epitope tagged rho constructs it is clear that a small fraction of all three proteins become localised to cellular membranes while the majority remain cytosolic. RhoA and rhoC localise to the plasma membrane, while rhoB localises to early endosomes and a pre-lysosomal compartment (Adamson et al., 1992). Interestingly rhoB is an immediate early response gene induced by growth factor stimulation of Rat fibroblasts while, rhoA and C are not (Jahner and Hunter, 1991).

**Cellular functions of rho proteins**

The first indication of the cellular function of rho came from the observation that microinjection of recombinant Val14-rho induced rapid changes in cell morphology. Activated rhoA induced actin polymerisation and contraction of the cell body, whereas microinjection of C3 transferase caused cells to round up, although they still remained attached to the substratum (Paterson et al., 1990). C3 and C2 transferase induce changes in the cytoskeleton of mammalian cells, disassembling the actin network and collapsing the intermediate filament system (Chardin et al., 1989; Weigers et al., 1991). More recently rho has been shown to directly regulate the formation of both actin stress fibres and focal adhesions. When activated rhoA is microinjected into serum-starved Swiss 3T3 cell (which lack stress fibres and focal adhesions), it rapidly stimulates the formation of actin stress fibres and focal adhesions. Moreover, stimulation of adhesions and stress fibres by extracellular factors such as the bioactive lipid LPA could be blocked by inhibiting rho (Ridley and Hall, 1992). This work essentially established rho as one of the central regulatory proteins of the actin cytoskeleton. Details of this signalling pathway will be discussed later.

Rho is fundamental to many other cellular events in a variety of cell types, such as neurite outgrowth in PC12 cells (Nishiki et al., 1990), where ADP ribosylation of rho inhibits LPA and thrombin induced neurite retraction and neuronal cell rounding (Jalink et al., 1994a). Significantly inhibition of rho inhibits aggregation of activated platelets and lymphocytes (Morii et al., 1992; Tominaga et al., 1993). Indeed in lymphocytes, it has being implicated in mediating the inside-out signal that enables the activated LFA-1 (T-cell receptor) to interact with ICAM-1 (Tominaga et al., 1993), with ADP ribosylation of rho ultimately inhibiting lymphocyte mediated cytotoxicity (Lang et al., 1992). In platelets it is thought to regulate the avidity of the $\alpha_{\text{Rco}}\beta_{\text{IIa}}$ integrin in aggregation (Morii et al., 1992). In S. cerevisiae, the RHO family of proteins have been shown to play an important role in budding, RHO1 and RHO3 co-operating to bring about bud enlargement (Yamochi et al., 1994). Other cellular phenomena have also been shown to involve rho. In KB cells, microinjection of C3 transferase inhibits both hepatocyte growth factor (HGF) and TPA induced membrane ruffling (Nishiyama et al., 1994), and suppresses the HGF-induced cell motility, an event also inhibited by microinjection of rhoGDI (Takaishi et al., 1994).
Interestingly, when overexpressed in serum-deprived NIH3T3 cells rho induces apoptosis (Jimenez et al., 1995). Finally by virtue of the control that rho exerts over actin and cytoskeletal organisation, is has been shown to be involved in bone resorption by osteoclasts (Zhang et al., 1995) and in secretion by mast cells (Norman et al., 1994; Price et al., 1995).

At the subcellular level rho proteins have been shown to regulate the activity of a number of signalling enzymes. It is well known that rhoA serves to activate tyrosine kinases that phosphorylate pp125Fak and paxillin (Rankin et al., 1994; Ridley and Hall, 1994; Seckl et al., 1995), but it has also been shown to stimulate a number of other types of signalling enzymes that generate lipid second messengers. Treatment of platelets with thrombin or GTPγS induces the production of PIP2 and PIP3, lipid second messengers that affect the actin cytoskeleton (Zhang et al., 1993; Carter et al., 1994). Upon activation of platelets, rho becomes rapidly recruited to the cytoskeleton. PI 3-kinase activity in platelet fractions could also be stimulated by incubation with GTPγS or addition of activated rhoA, but inhibited by C3 transferase (Zhang et al., 1993). The mechanism by which rho activates PI 3-kinase in platelets is unclear. An increase in PI 3-kinase activity has also been detected in Swiss 3T3 cells treated with LPA. Stimulated cells exhibited an increased tyrosine phosphorylation of the p85 subunit of PI 3-kinase, and showed an increase in PIP2 and PIP3 which was inhibited by C3 transferase (Kumagai et al., 1993). Rho has been implicated in regulating another lipid modifying enzyme the PIP 5-kinase, which phosphorylates PI(4)P or PI(3,4)P2 to generate PI(4,5)P2 and PI(3,4,5)P3 respectively. The activity of this enzyme in a cell free system can be stimulated by the addition of rhoA, and inhibited in cells by microinjection of C3 transferase (Chong et al., 1994). Rho also regulates a third lipid modifying enzyme phospholipase D (PLD), which hydrolyses phosphatidyl choline to produce phosphatidic acid (PA) and choline. In rat liver membrane preparations the activity of PLD can be stimulated by the addition of GTPγS in a manner that is inhibited by washing the membranes with rhoGDI, but re-established upon the readdition of activated rho (Malcolm et al., 1994). The generation of acidic lipid messengers such as PA, PIP2 and PIP3 are thought to regulate release of actin sequestered monomers and the release of barbed ends, stimulating the formation of actin filaments (Stossel, 1993; Machesky and Pollard, 1993). It is possible that rho proteins utilise the modification of lipid metabolites to exert positive controls over the organisation of the actin cytoskeleton.

Role of rho in Swiss 3T3 fibroblasts
In Swiss 3T3 cells rho proteins can be activated by growth factor such as PDGF or mitogenic soluble products such as the bioactive lipid LPA or the neuropeptide bombesin. When cells are stimulated with PDGF, a sequential signal is transmitted by ras and rac to activate rho, as discussed below. LPA and bombesin exert a more direct and efficient
stimulation of rho. The pathway by which LPA exerts its effects on the cytoskeleton has been primarily dissected using Swiss 3T3 fibroblasts and is summarised in Fig 1.12. In serum-starved Swiss 3T3 cells LPA induces the rapid formation of focal adhesions and stress fibres and the tyrosine phosphorylation of a number of proteins including pp125Fak and paxillin (Barry and Critchley, 1994; Ridley and Hall, 1994). LPA interacts with a heterotrimeric G-protein receptor (LPA-R), thought to be a 39 kDa protein identified by chemical cross linking (van der Bend et al., 1992). LPA-R-mediated stimulation of rhoA is dependent on the action of a tyrphostin 25 sensitive tyrosine kinase. Cells treated with the tyrosine kinase inhibitor tyrphostin 25 were unable to respond to LPA, but formed actin stress fibres and focal adhesions upon microinjection of activated rhoA, indicating that the kinase lies upstream of rhoA (Nobes et al., 1995). How this kinase is stimulated by LPA is unknown. A second, genistein-sensitive tyrosine kinase lies downstream of rhoA. Treatment of cells with genistein again inhibited the LPA-mediated formation of actin stress fibres and focal adhesions. However in this case, microinjection of rhoA failed to produce a response indicating that the kinase lay downstream of rhoA (Ridley and Hall, 1994). Once the signal has passed to the genistein-sensitive tyrosine kinase the signal bifurcates. Treatment of cells with cytochalasin D, followed by microinjection of rhoA, stimulates the formation of vinculin-containing focal adhesions that are not associated with actin stress fibres. Conversely, treatment with the broad range kinase inhibitor staurosporine inhibits focal adhesion formation upon microinjection of rhoA, although the formation of actin stress fibres is evident (Nobes and Hall, 1995b). This stimulation of actin polymerisation may be mediated through the activation of PIP 5-kinase (Chong et al., 1994). The identity of the various tyrosine kinases which operate in this pathway are unknown as are the mechanisms of activation. Identifying these kinases would greatly increase our understanding of adhesion regulation and probably give some insight into how various signalling pathways interact. A summary of these signalling events is shown in Fig. 1.11.

1.7.4 Signalling by rac and cdc42

Two rac proteins, rac1 and rac2 have been discovered to date, rac1 being ubiquitously expressed while rac2 is expressed primarily in cell of myeloid lineage and is the predominant isoform in neutrophils (Heyworth et al., 1994). They are 95% identical at the amino acid level, and 60% identical to rho, with the same domain structure. Mutations have proven invaluable in studying the cellular functions of the rac proteins. They can be constitutively activated by a glycine to valine mutation at position 12, a dominant negative mutant is created by a serine to asparagine mutation at position 17, while the effector function of the protein can be negated by a threonine to alanine mutation at residue 35 (Ridley et al., 1992). Rac1 regulates aspects of cytoskeletal organisation (see later) (Ridley et al., 1992), while rac2 is involved in activation of the NADPH oxidase system in
phagocytes (Abo et al., 1991; Knaus et al., 1992) where it is required for recruiting the phox proteins to the membrane (Benna et al., 1994). The NADPH oxidase system produces superoxide anions in response to a number of inflammatory stimuli. The superoxide anions are released into phagosomes containing engulfed bacteria, destroying them with free radicals.

Rac1 has been implicated in a number of important cellular processes. In mast cells rac has been shown to be essential for the organisation of the cortical actin network following activation, while microinjection of activated rac (like rhoA), enhanced the regulated secretion from these cells (Norman et al., 1994; Price et al., 1995). In permeabilised platelets, both constitutively active rac and activation of the thrombin receptor have both been shown to uncap actin filament barbed ends through phosphoinositide synthesis. The same uncapping of actin filament barbed ends can be mimicked by acidic phospholipids such as PIP, PIP2 (3,4) and (4,5), and PIP3 (Hartwig et al., 1995). Rac could possibly activate actin polymerisation through production of phosphoinositide lipids which uncap F-actin filament barbed ends.

cdc42
Cdc42 was originally discovered as a rho-related gene (CDC42) required for bud formation and cell polarity in the budding yeast S. cerevisiae (Adams et al., 1990; Johnson and Pringle, 1990). Two very closely related human homologues of yeast CDC42 have been cloned, named G25K and cdc42Hs. The human proteins are identical to the yeast protein except for an 8 amino acid segment in the C-terminus of the protein, and can rescue the deficiency of CDC42 when expressed in yeast (Munemitsu et al., 1990; Shinjo et al., 1990; Michetti et al., 1991). Like its fellow rho family members, cdc42 has also been implicated in playing a crucial role in the regulation of specific cytoskeletal structures, namely actin microspikes or filopodia (Kozma et al., 1995; Nobes and Hall, 1995b). Also like rac and rho, cdc42 can be mutated at amino acid 12 to give a constitutively active protein, and at amino acid 17 to create a dominant negative mutant.

Effects of rac and cdc42 on the actin cytoskeleton
Rac was first shown to control the formation of membrane ruffles in serum-starved Swiss 3T3 cells, and is thought to play a role in organising the cortical actin network. Membrane ruffling is caused by the polymerisation of actin under the membrane, a phenomena induced by treatment of many cell types with growth factors or phorbol esters which activate PKC (Nister et al., 1988; Ridley et al., 1992). Treatment of serum-starved Swiss 3T3 cells with PDGF and TPA induced the formation of membrane ruffles, which could be mimicked by microinjection of the constitutively active mutant of rac. Conversely, membrane ruffling could be inhibited by microinjection of the dominant negative mutant of rac (Ridley et al., 1992). More recently the human cdc42 protein has been shown to
regulate the formation of actin microspikes or filopodia in Swiss 3T3 cells (Kozma et al., 1995; Nobes and Hall, 1995b), actin rich structures which have important sensory functions in migrating or spreading Swiss 3T3 cells (Albrecht-Buehler, 1976) and in the growth cone of neuronal cells (Hynes and Lander, 1992; Chien et al., 1993). Interestingly, microinjection of cdc42 into Swiss 3T3 was shown induce the disassembly of actin stress fibres, and the accumulation of punctate actin, the significance of which is unclear. The formation of filopodia could only be mimicked by treating cells with bradykinin (Kozma et al., 1995), though this has not been observed in other studies. Cdc42 induced filopodia and rac associated membrane ruffles both contained small clusters of vinculin, paxillin and pp125Fak (Nobes and Hall, 1995b) indicating that they can also induce the formation of small adhesive complexes. The specific downstream events leading to ruffling and filopodia formation are however poorly understood. The effects of cdc42 and rac on the cytoskeleton are summarised in Fig. 1.11.

The rho-related small GTP-binding proteins appear to regulate adhesion and the actin cytoskeleton in a co-ordinated manner. As a result, one downstream effect of cdc42 activation is the activation of rac, while activation of rac leads to activation of rho (Nobes and Hall, 1995b; Nobes and Hall, 1995a). Indeed, upon microinjection of cdc42 into Swiss 3T3 cells, time lapse photography showed the once the filopodia had formed the space between the individual filopodia was filled in by the formation of lamellapodia in a rac-dependent manner (Nobes and Hall, 1995b). Microinjection of rac into Swiss 3T3 cells stimulates the formation of some actin stress fibres indicating a signal from rac to rhoA (Ridley et al., 1992). Rac may activate rho by the production of leukotrienes. In Swiss 3T3 fibroblasts, rac induces the production of leukotrienes from arachadonic acid through activation of 5-lipoxygenase. This enzyme may be stimulated either by EGF or the introduction of activated rac. The leukotrienes produced, in turn, induce the formation of stress fibres in a rho-dependent manner (Peppelenbosch et al., 1995). Significantly 5-lipoxygenase has a SH3-binding site motif capable of interacting with the SH3 domain of grb2, and has been shown to interact with both α-actinin and actin (Lepley and Fitzpatrick, 1994). Although activation of this protein and the production of lipid messengers may explain how signals are passed from rac to rho, it is still unclear how cdc42 activates rac (Summarised Fig. 1.11).

**Targets and regulators of rac and cdc42**

PDGF activates rac via PI 3-kinase, as treatment of cells with the PI 3-kinase inhibitor wortmannin (Nakanishi et al., 1994) inhibits PDGF-induced membrane ruffling (Nobes et al., 1995). This activation has been shown to be directly dependent on the activation of PI 3-kinase (Hawkins et al., 1995), perhaps due to the production of the lipid metabolite PIP3 which may uncouple the rac-rhoGDI interaction allowing rac to become activated (Parker, 1995). An upstream activator for cdc42 has also been identified and cloned. This is a
Figure 1.12. Regulation of actin containing cytoskeletal structures by cdc42, rac and rho small GTP binding proteins. cdc42 stimulates the formation of filopodia, though the upstream regulators are unknown. rac stimulates the formation of lamellipodia. Rac can be stimulated in three ways. By activation of cdc42 or ras, and by PDGF through activation of PI3 kinase. Rho stimulates the formation of focal adhesions and actin stress fibres. Rho is regulated upstream either by rac activation of 5-lipoxygenase or through LPA-mediated activation of a tyrphostin sensitive tyrosine kinase. The downstream effects of rho appear to be mediated initially by a genistein sensitive tyrosine kinase. Adhesion formation is dependent on a staurosporine sensitive kinase while stress fibre formation may well be mediated through activation of PIP 5-kinase. Modified from Nobes and Hall (1995).
novel hippocampal tyrosine kinase p120ACK which appears to inhibit both the GAP-induced and intrinsic GTPase activity of cdc42 via a region in its C-terminus just after an SH3 domain (Manser et al., 1993). Whether this kinase is ubiquitous in its control of cdc42 is not known.

Direct associations have been demonstrated between rac and PIP 5-kinase, and both cdc42 and rac with PI 3-kinase. Fusion proteins corresponding to both rac and cdc42 co-precipitated the respective enzymatic activities, while a rhoA fusion protein precipitates did not. The co-precipitation of active enzyme with fusion proteins also appeared to be GTP dependent (Tolias et al., 1995), implying that rho proteins may mediate their effects in the cytoskeleton through the production of lipid metabolites. However, an in vivo association of PI 3-kinases with rac was induced by PDGF treatment, implying that PI3-kinase was downstream of rac. It is hard to reconcile that a protein required for the activation of rac (Nobes et al., 1995) in vivo could also be rac's downstream effector. The same may not be true for cdc42 which activates PI 3-kinase in vitro by binding to the p85 subunit (Zheng et al., 1994). Both rac and cdc42 also activate a serine/threonine kinase p65PAK, a ubiquitously expressed protein originally identified and cloned from brain (Manser et al., 1994). Rac and cdc42 interact with p65PAK within its regulatory domain, though whether they exert any effect on the cytoskeleton through this association is again unclear.

1.7.5 Regulation of rho family members

As described above, small GTP binding proteins are regulated by a number of factors, GAPs, GDIs and exchange factors. A list of the potential rho family regulators and their targets is shown in Table 1.4. These factors can all potentially have effects on the cytoskeleton. Indeed the disruption of the actin cytoskeleton by the N-terminus of rasGAP has already been discussed (McGlade et al., 1993). Tight control on the small GTP-binding proteins is exerted by the various regulatory protein, exemplified by the fact that one nucleotide exchange factor for rho and cdc42, dbl was originally identified because it is an oncogene (Hart et al., 1994), while two other potential rho-family exchange factor have been cloned as the genes responsible for diseased states (Habets et al., 1994; Pasteris et al., 1994).

GTPase activating proteins

A number of GTPase activating proteins for the rho family members have now been identified. The first was p50 rhoGAP, initially discovered as a 29 kDa protein in spleen extract that possessed rhoGAP activity (Garrett et al., 1989). Cloning of the cDNA revealed this to be a 50 kDa protein that also contains a proline-rich SH3-binding domain. The 29 kDa protein originally identified was in fact a C-terminal proteolytic fragment of the full length rhoGAP (Lancaster et al., 1994). Sequences homologous to the rhoGAP
domain are found in a wide variety of proteins many of which are large and multifunctional (Lamarche and Hall, 1994). p50rhoGAP shows activity against rho, rac and cdc42/G25K in vitro, though its preferred substrate was cdc42 (Ridley et al., 1993). Cloning of a human cdc42GAP has also been reported, which also contains a functional SH3-binding domain (Barford et al., 1993), and is identical to rhoGAP. The SH3-binding domain of the protein was shown to interact with both P85 and v-src (Barford et al., 1993).

The genuine rhoGAP appears to be p190, the rasGAP associated protein, which has previously been shown to possess rhoGAP activity in vitro (Settleman et al., 1992a), and possesses little activity against rac and G25K (Ridley et al., 1993). In vivo, both the GAP domains of p50rhoGAP and p190rhoGAP inhibit LPA and bombesin-mediated stress fibre formation, but not PDGF-mediated membrane ruffling (Ridley et al., 1993). Interestingly, p190rhoGAP has itself been reported to bind GTP and exhibits a GTPase activity stimulated by an unknown factor in cell lysates (Foster et al., 1994). One protein that inhibits PDGF membrane ruffling when microinjected is the GAP domain of BCR (Ridley et al., 1993), which has previously been shown to possess GAP activity against rac in vitro (Diekmann et al., 1991). BCR is a large 143 kDa protein that contains a serine/threonine kinase domain at its N-terminus, and a GAP domain at the C-terminus. It forms part of the BCR-ABL oncoprotein generated by a chromosome translocation in chronic myelogenous leukaemia (Lamarche and Hall, 1994). A number of proteins possess GAP activity against rac: ABR a protein closely (65% at amino acid level) related to BCR; the N- and P-chimaerins, which are expressed at the highest levels in brain and testis respectively (Leung et al., 1994; Herrera and Shivers, 1995; Yuan et al., 1995); and more recently an SH3 domain-containing protein 3BP-1. 3BP-1 shows racGAP activity in vitro and microinjection blocks PDGF-induced membrane ruffling (Cicchetti et al., 1995). Interestingly the p85α and β subunits of PI 3-kinase also contain GAP homology regions but they are not known to be functional.

A functional link between the cytoskeleton and the regulation of rho-like proteins has come with the cloning of myr-5 a novel myosin found in rat. The protein is capable of interacting with actin filaments and also possesses GAP activity specifically against the rho family GTPases (Reinhard et al., 1995). To complicate the picture further, a novel 122 kDa rho specific rhoGAP has also been cloned that is capable of stimulating the PIP2 hydrolysing activity of phospholipase C-β (Homma and Emori, 1995).

Guanine nucleotide dissociation inhibitors

Although the GAPs have been studied in detail less is known about the function of the GDI protein. Overexpression of rhoGDI in cells disrupts the actin cytoskeleton (Jeffers et al., 1993), implying that it does indeed play an important role in regulating rho activity in vivo. RhoGDI is important in maintaining rho-like proteins in an inactive state, inhibiting release of GDP. RhoGDI was originally identified as a 27 kDa protein in brain cytosol (Fukumoto
et al., 1990), and appears to be ubiquitously expressed. As well as interacting with rho, rhoGDI also appears to interact with p21rac and G25K/cdc42. The rhoGDI-GTP-binding protein complex is cytosolic, the interaction with rho requiring post-translational modification at the C-terminus (Hori et al., 1991). The interaction with the GDP-bound protein is thought to be mediated by rhoGDI binding the prenylation moiety of the molecule (Hancock and Hall, 1993), perhaps by insertion into a hydrophobic pocket within the centre of the GDI (Takai et al., 1995). RhoGDI has been reported to have a ten times higher affinity for the GDP-bound form of rho and rac than, the GTP-bound form (Sasaki et al., 1993). In vitro GTP-bound rac and rho are resistant to the action of GAP proteins, the rac-GTP-GDI complex being stronger than that formed between rac-GDP-GDI (Hancock and Hall, 1993). For rac, rhoGDI may serve some extra function than just as a negative regulator. Rho-GDI can however compete membrane bound rho from the membrane translocating it to the cytosol (Malcolm et al., 1994), probably binding once the protein has hydrolysed GTP. Two other rhoGDI like protein have been identified LyGDI expressed in lymphocytes and D4 expressed in haemopoietic cells (Ridley, 1994).

Guanine nucleotide exchange factors/dissociation stimulators
The third group of rho regulators are the GDS proteins, though little is known about GDS proteins that act specifically on rho proteins. A number of exchange factors have been described for ras, such as sos and rasGRF, which stimulate the dissociation of GDP and hence exchange for GTP. The mechanism by which GTP exchange is achieved is not known although a number of potential GDS exchange factors have been described for the rho family. A protein with rhoGDS activity was first purified from brain cytosol (Isomura et al., 1990), though no rho specific rhoGDS has been cloned. GDS proteins have been described for cdc42 and rac, which also show some activity against rho. The product of the dbi oncogene has GDS activity for cdc42 and rhoA, but not rac1 and TC10 another rho family member, the activity against cdc42 being the highest. Its exchange stimulating domain is contained within a 238 amino acid region (Hart et al., 1994). The only rac exchange factor described to date is the smgGDS which also shows activity against rho (Chuang et al., 1994). Sequences homologous to the 238 amino acid region in dbi have been identified in a number of proteins leading to the suggestion that they act as exchange factors for the rho family. These include ber and the oncogenes ect2 and vav. Indeed ect2 protein has been shown to interact with rac1, rhoA and rhoC (Miki et al., 1993). However vav possesses exchange activity against ras and not the rho-related proteins (Gulbins et al., 1993), perhaps signifying that dbi homology regions are not so specific for the rho-related proteins.

A novel oncogene, ost, cloned from an osteosarcoma shows nucleotide exchange activity against rhoA and cdc42 and also appears to have effector activity for rac1. The gene product is 100 kDa, contains a dbi homology region and has the potential to link rho and
cdc42 (Horii et al., 1994). Finally two disease related genes recently cloned contain dbl homology regions. The first is the Tiam-1 gene identified as an invasion inducing gene disruption of which is associated with the invasive capacity of certain T lymphoma variants (Habets et al., 1994). The second is a gene associated with the developmental abnormality Faciogenital Dysplasia or Aarskog-Scott Syndrome and occurs through an X-linked genetic defect, which results in short stature and facial, skeletal and urogenital abnormalities. The gene contains sequence homologous to rho exchange factors and also contains two potential SH3 domains (Pasteris et al., 1994).

A representation of an idealised GTP cycle for the rho proteins is shown in Fig. 1.13.

1.7.6 Rho-related proteins regulate transformation, the cell cycle and gene expression

It is well documented that ras GTPases are associated with the development of malignant phenotypes, but more recently evidence has accumulated that rho-like GTPases play a role in ras mediated transformation of cells (Khosravi et al., 1995; Qui et al., 1995). Rac1 was the first shown to be an essential component of ras transformation. Expression of a dominant negative rac in a ras-transformed cell line inhibited transformation, but was ineffective in cells expressing a constitutively activated raf kinase. Moreover cells expressing only high levels of activated rac had the ability to grow in soft agar and form tumours when implanted in nude mice (Qui et al., 1995). Furthermore, rhoA and rac mutants, showing a weak transforming ability, compliment a weakly transforming ras mutant resulting in a 35 fold increase in potency. The same mutants also enhanced the phenotypes of ras-transformed mutants (Khosravi et al., 1995). The synergistic nature of the transformation effects is consistent with the activation of two separate signalling pathways. Indeed rhoA, rac and cdc42 have each been shown to play an essential role in driving cells through G1 of the cell cycle. Microinjection of rhoA, rac and cdc42 into quiescent cells stimulates the cell cycle through G1 and drives DNA synthesis. Serum-induced DNA synthesis and cell cycle progression could be inhibited by inhibiting each of these proteins. The DNA synthesis induced by rac and cdc42 does not occur through the conventional ras linked MAP kinase pathway, but rather through activation of a second related MAP kinase, the JNK (jun N-terminal kinase) pathway activated in response to stress stimuli (Olson et al., 1995). Both activated cdc42 and rac1 activate the JNK signalling cascade and the transcriptional activity of the transcription factor c-jun, which was inhibited by inhibitory elements (GDI) and activated by exchange factors (Coso et al., 1995). Cdc42 and rac may play a role in linking the ras-raf-MAP kinase cascade to the JNK pathway, as well mediating activation of stress responses (Minden et al., 1995). Both these GTPase-activated pathways appear to converge on the serum response element (SRE), a nucleotide sequence within promoters that regulates expression of immediate early response genes. Activation of transcription from the SRE has been suggested to involve phosphorylation of another transcription factor Elk-1 (Whitmarsh et al., 1995).
### Guanine Nucleotide Dissociation Inhibitors

<table>
<thead>
<tr>
<th>GTPase activating proteins</th>
<th>Substrate</th>
<th>Expression</th>
</tr>
</thead>
<tbody>
<tr>
<td>p50rhoGAP</td>
<td>rho, rac, cdc42*</td>
<td>ubiquitous</td>
</tr>
<tr>
<td>Bcr (exhibits ser/thr kinase activity)</td>
<td>rac, cdc42</td>
<td>Bcr-abl fusion formed in some leukemias</td>
</tr>
<tr>
<td>Abbr</td>
<td>rac, cdc42</td>
<td>Homologous to bcr</td>
</tr>
<tr>
<td>N-chimaerin</td>
<td>rac</td>
<td>Brain specific</td>
</tr>
<tr>
<td>b-chimaerin</td>
<td>rac</td>
<td>Testes specific</td>
</tr>
<tr>
<td>p190 (exhibits GTPase activity)</td>
<td>rho*, cdc42</td>
<td>Ubiquitous</td>
</tr>
<tr>
<td>p85α (putative)</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>p85β (putative)</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>3BP-1</td>
<td>rac</td>
<td>Low expression most tissues</td>
</tr>
<tr>
<td>Myr 5 (binds actin)</td>
<td>rho, cdc42, rac§</td>
<td>widespread</td>
</tr>
<tr>
<td>p122 (stimulates PLCγ)</td>
<td>rho</td>
<td>brain?</td>
</tr>
</tbody>
</table>

### Guanine Nucleotide Exchange Factors

<table>
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<tr>
<th>GTPase activating proteins</th>
<th>Substrate</th>
<th>Expression</th>
</tr>
</thead>
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<tr>
<td>rhoGDI</td>
<td>rho, rac, cdc42</td>
<td>Ubiquitous</td>
</tr>
<tr>
<td>LyGDI</td>
<td>rho</td>
<td>Lymphocytes</td>
</tr>
<tr>
<td>D4</td>
<td>rho</td>
<td>Haemopoietic cells</td>
</tr>
</tbody>
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<table>
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<tr>
<th>Guanine Nucleotide Dissociation Inhibitors</th>
<th>Substrate</th>
<th>Expression</th>
</tr>
</thead>
<tbody>
<tr>
<td>Smg-GDS</td>
<td>rho, rac</td>
<td>Ubiquitous</td>
</tr>
<tr>
<td>Dbl</td>
<td>rho, rac, cdc42</td>
<td>Brain, adrenal glands, gonads</td>
</tr>
<tr>
<td>Bcr</td>
<td>-</td>
<td>Dbl homology domain found in Bcr-abl in some leukemias</td>
</tr>
<tr>
<td>Ect2</td>
<td>rho, rac</td>
<td>Kidney, liver, spleen testis</td>
</tr>
<tr>
<td>Ost</td>
<td>rho, cdc42</td>
<td>Isolated from osteosarcoma; high in brain</td>
</tr>
<tr>
<td>Vav</td>
<td>-</td>
<td>High message levels during development.</td>
</tr>
<tr>
<td>Faciogenital dysplasia gene</td>
<td></td>
<td>message detected brains and testes</td>
</tr>
<tr>
<td>Tiam-1</td>
<td>predicted 107</td>
<td>contains dhl homology region</td>
</tr>
<tr>
<td>Sos (ras exchange factor)</td>
<td>putative</td>
<td></td>
</tr>
</tbody>
</table>

### Table 1.4. Potential regulators of mammalian rho-related proteins.

Summary of putative regulatory protein of the mammalian rho family member. * represents preferred substrate, § represents weak activity against that proteins. Table assembled from information contained within a number of reviews (Lamarche and Hall, 1994; Ridley, 1994; Takai et al., 1995), addition information was obtained from papers specifically cited in the text.
The GTPase cycle.

Figure 1.13. Representation of a possible GTPase cycle of rhoA. The general principles of the cycle apply to both ras and rho-related proteins discussed in this section. GTP bound rhoA is stimulated by GAP (GTPase activating protein), and hydrolyses GTP. It is then competed from the membrane bound to GDI (guanine nucleotide dissociation inhibitor), and remains complexed in the cytoplasm, bound to GDP, until acted upon by a GEF/GDS (guanine nucleotide exchange factor/guanine nucleotide dissociation stimulator). RhoA then becomes GTP bound and is translocated to the membrane either complexed with the GEF/GDS or bound to another factor X. Once at the membrane in the GTP bound form rhoA generates a signal until the GTP is hydrolysed.
Rho does not mediate an effect on either of these pathways but rather functions in a novel signalling pathway capable of stimulating c-fos expression through activation of heterotrimeric G-protein receptors, distinct from signals induced by activation of receptor tyrosine kinases (Hill and Treisman, 1995; Hill et al., 1995).

In summary small GTP-binding proteins of the rho family appear to regulate the formation of multimeric signalling/structural complexes such as adhesion complexes and play crucial role in mediating activation of pathways regulating gene expression.

1.8 Lysophosphatidic acid (LPA)

LPA is a water soluble mitogenic bioactive lipid that is capable of stimulating adhesive responses, and the tyrosine phosphorylation of cellular proteins in a number of cell lines (Ridley and Hall, 1992; Barry and Critchley, 1994; Chranowska-Wodnicka and Burridge, 1994; Hordijk et al., 1994; Ridley and Hall, 1994; Saville et al., 1994; Seufferlein and Rozengurt, 1994), and neurite retraction in differentiated PC12 cells (Jalink et al., 1994a). It induces numerous physiological and cellular process, including aggregation of platelets, contraction of smooth muscle cells (Moolnaar, 1994), and promotion of the invasive capacity of tumours into basement layers of mesothelial cells (Imamura et al., 1993). LPA is produced as a result of a number of stimuli. Upon platelet stimulation it is synthesised and released from the cells either by vesicular transport or diffusion (Eichholtz et al., 1993). Interestingly LPA is also produced in small amounts upon growth factor stimulation of fibroblasts, and is liberated from wounded fibroblasts (Moolnaar, 1994). It is present in serum at a concentration of 1-5 μM, but probably at higher local concentrations at sites of wounding (Jalink et al., 1994b). Most lipids have a short half life once in the blood stream, but LPA appears to be protected from degradation through association with a number of serum proteins including albumin (Thumbser et al., 1994), and two unidentified proteins of 28 and 17 kDa (Moolnaar, 1994). De novo synthesis of LPA occurs in the ER membrane by the action of phospholipase A$_2$ (PLA$_2$) on phosphatidic acid (Jalink et al., 1994b). PLA$_2$ is also responsible for the production of prostaglandins and leukotrienes which also have effects on adhesion as discussed earlier.

At the sub-cellular level LPA exerts an effect on a number of distinct signalling pathways. The LPA receptor is thought to be a heterotrimeric seven transmembrane pass G-protein receptor of 30 kDa (van der Bend et al., 1992), activation stimulating classic G-protein signalling pathways. The first is through a pertussis toxin insensitive G-protein Gq activating phospholipase C (PLC), which leads to PKC activation and Ca$^{2+}$ mobilisation (Jalink et al., 1990). LPA has also been implicated in activation of PLD activity, in a pertussis toxin sensitive and PKC dependent manner, which is associated with some actin polymerisation in IIC9 fibroblasts (Ha et al., 1994). The second pathway is activated through the pertussis toxin sensitive G-protein G$\gamma$ which leads to reduced adenylate
Figure 1.14. Signalling events associated with stimulation of the LPA receptor. (A simplified model modified in part from Moolenaar, 1994). AC - adenylate cyclase; PLC - phospholipase C; PKC - protein kinase C; MEK - MAP kinase kinase; MAPK - MAP kinase; DAG - diacyl glycerol; IP3 - inositol triphosphate. LPA stimulates PLC and rho-dependent tyrosine phosphorylation and cytoskeletal changes through a pertussis toxin insensitive G-protein, presumably Gq, although participation of additional G proteins has not been ruled out. LPA inhibits AC and stimulates the ras-MAPK pathway activity through pertussis toxin sensitive Gi. Since a rise in cAMP inhibits the ras-MAPK pathway apparently at the level of raf, a Gi mediated fall in cAMP levels might positively influence ras-raf signalling. Whether inhibition of AC and ras activation are mediated by the same Gi protein is not clear. Very little is known about the upstream and downstream effectors of rho, though rho appears to be coupled to the LPA receptor via a tyrphostin 25 sensitive kinase. It remains possible that rho-dependent tyrosine phosphorylation and cytoskeletal changes represent changes in parallel rather than sequential events.
cyclase activity. G\textsubscript{i} action also stimulates the rapid accumulation of GTP bound ras, leading to activation of the MAP kinase cascade (van Corven et al., 1993; Hordijk et al., 1994). The activation of ras is reported to be mediated by a genistein and staurosporine sensitive tyrosine kinase (Hordijk et al., 1994). This activation of the MAP kinase pathway is likely to account for the mitogenic properties of LPA.

The effect of LPA on the actin cytoskeleton and focal adhesions has been shown to be independent of the classical signalling pathways associated with LPA activation of cells (Chrzanzowska-Wodnicka and Burridge, 1994; Ridley and Hall, 1994), although inhibition of PLC appears to inhibit actin polymerisation (Chrzanzowska-Wodnicka and Burridge, 1994). The LPA receptor appears to be coupled to the rho pathway via a herbimycin sensitive tyrosine kinase (Nobes et al., 1995), perhaps activated as a result of activation of G\textsubscript{q}. Similar signalling events are elicited when cells are stimulated with neuropeptides such as bombesin and vasopressin, which also bind to receptors coupled to heterotrimeric G-protein (Zachary et al., 1993).

1.9 Regulation of actin polymerisation

When considering the regulation of cell adhesion, attention must be given to the process of actin polymerisation. Actin itself is an ATPase that exists as 43 kDa monomers which will spontaneously form filaments above the critical concentration of 0.2 \mu M (Pantaloni et al., 1985). Actin filaments consist of a barbed end and a pointed, with ATP- rather than ADP-actin monomers being added preferentially to the barbed end of the filament. Although not the preferred reaction, ADP monomers can be added to the barbed end and monomers can also be incorporated into the pointed end of the filament, but the critical concentration for both these events is higher than that of ATP-actin addition to the barbed end. Once an ATP bound actin monomer is incorporated into the growing filament the ATP is eventually hydrolysed to yield ADP-actin. The ADP-actin may be eventually released from the pointed end as the fibre cycles, though release from the barbed end is quicker and is the mechanism of monomer exit when rapid disassembly occurs (Korn et al., 1987; Stossel, 1993). Due to the rate of ATP hydrolysis, actin filaments are heterogeneous consisting of ATP- and ADP-bound actin monomers. The formation of filamentous actin is regulated in a number of ways. By the ATPase and ATP/ADP state of individual actin monomers and by two classes of actin-binding proteins, namely monomeric actin-binding proteins and actin-capping proteins and actin-capping/severing proteins. Once the actin has nucleated and formed an F-actin filament the filaments are organised and bundled by a number of proteins to form the actin structures recognisable under the microscope, for example the actin stress fibre. Proteins that organise actin filaments into fibres and cables include fimbrin/plastin, \alpha-actinin, spectrin, fodrin, dystrophin, utrophin, ABP-120, and filamin (Matsudaira, 1991).
Proteins that regulate actin

There are two alternative models which attempt to explain mechanisms by which actin filaments can be rapidly generated. The first is via de novo synthesis of F-actin. This process is mediated by actin nucleating proteins which create nucleated F-actin from activated actin monomers. The filament then grows from the barbed end of the nucleated actin. The second is the formation of filaments by the addition of monomers to preformed nucleation centres. This is achieved by the release of sequestered barbed ends, by the creation of new barbed ends by the severing of existing filament. In both models, the growth of the actin filament relies on the availability of activated monomeric actin which can polymerise at the free barbed end of the filament. These models have been developed mainly from observations made in platelets. Although actin regulation in platelets is unlikely to be identical to the process in fibroblasts, it provides the best example with which to introduce the concepts and proteins that regulate F-actin formation.

Monomeric actin-binding proteins

The concentration of G-actin within the cell is over 200 μM, far exceeding the critical concentration for spontaneous polymerisation. Actin monomer binding proteins sequester actin monomers and prevent them from polymerising. Three such proteins are profilin (12-15 kDa), thymosin-β4 (5 kDa) and ADF/cofilin (15-19 kDa) which are all thought to bind the barbed end of the actin monomer (Sun et al., 1995). Disruption of the profilin gene resulted in defects in actin-dependent processes in a number of organisms (Haarer et al., 1990; Verheyen and Cooley, 1994). Profilin has dual effects on actin which depend very much on the context. Microinjection of profilin alone into animal cells depolymerises actin while injection of actin-profilin complexes induces actin polymerisation (Cao et al., 1992). It appears enriched in areas of active polymerisation such as the leading edge of migrating cells. As well as binding monomeric actin, profilin also acts as an ATP exchange factor binding ADP-actin monomers and promoting the exchange of ADP for ATP (Goldschmidt-Clermont et al., 1992). An interesting additional role of profilin is that it sequesters PIP2 in resting cells, preventing hydrolysis by PLC isoforms (Goldschmidt-Clermont et al., 1991). Upon tyrosine phosphorylation, PLC can access the profilin bound PIP2 (Goldschmidt-Clermont et al., 1991). The interaction of profilin with PIP2 inhibits the interaction of profilin with actin monomers (Lassing and Lindberg, 1985). High local concentrations of PIP2 may therefore result in the liberation of actin monomers.

The true actin sequestering proteins are the thymosin family of low molecular weight proteins (Sun et al., 1995). The best characterised is thymosin β4 which binds ATP-actin with an affinity similar to that of the critical concentration for actin polymerisation in a 1:1 ratio (Yu et al., 1993). The binding occurs with an affinity which is 100 fold higher for ATP-actin than for ADP-actin, and appears to inhibit nucleotide exchange when bound to ADP-actin (Carlier et al., 1993). This implies that thymosin β4 sequesters the "active"
actin monomers in the cell. The sequestration of actin by thymosin β4 is a passive process regulated only by the concentration of free actin monomer in the cell. In the cellular context, increases in thymosin β4 tend to depolymerise more unstable actin structures (Sanders et al., 1992; Yu et al., 1994).

Profilin and thymosin β4 co-ordinate to regulate the amount of active G-actin available for addition to the barbed ends of growing filaments. In vitro, profilin reduces the critical concentration of actin required for polymerisation at the barbed end from 0.2 μM down to 0.007 μM, which is further enhanced in the presence of thymosin β4 (Pantaloni and Carlier, 1993). Presumably as free actin monomer are used up and shuttled into filaments by profilin, the local concentrations of free actin falls resulting in the release of active actin sequestered by thymosin β4. Profilin may also utilise its nucleotide exchange activity to regenerate ATP-actin under conditions of rapid actin depolymerisation/polymerisation.

The third class of monomeric actin binding protein is the ADF/cofilin family. At pH<7.1 they decorate actin filaments, binding monomers on a one to one (actin monomer) basis displacing tropomyosin, myosin and the villin-head piece from filaments. Upon alkalization of the cytoplasm (pH>7.1) they bind actin monomers to inhibit nucleotide exchange and polymerisation (Sun et al., 1995). They also sever actin filaments, to generate uncapped barbed ends, increasing the potential sites for actin polymerisation through profilin-mediated monomer addition (Hawkins et al., 1993).

**Actin capping; actin capping/severing proteins**

The F-actin capping proteins regulate the availability of barbed ends for monomer addition. F-actin capping proteins preclude the barbed end of the actin filaments preventing or regulating monomer addition to the barbed end (Hartwig, 1992). Two proteins studied in most detail are gelsolin (83 kDa) and capZ (35 and 32 kDa) (Weeds and Maciver, 1993) In the resting platelet, the polymerised actin is in numerous short actin filaments capped by either capZ or gelsolin distributed abutting the membrane and organised by an actin crosslinking protein ABP280 (Barkalow and Hartwig, 1995). Large pools of both gelsolin and capZ are also free in the cytoplasm. Activation of platelets results in a rise in intracellular calcium and activation of the actin severing function of gelsolin. The mobilisation of actin during the platelet response involves the initial formation of small actin filaments, mediated by the actin severing function of gelsolin (Kwaikowski et al., 1989) The uncapping of gelsolin and capZ barbed ends, and the activation of the actin severing function of gelsolin creates numerous barbed ends which have the potential to act as nucleating centres for the growth of actin filaments through the addition of activated actin monomers (Hartwig, 1992). Indeed platelets lacking gelsolin do not exhibit this initial severing phenomenon (Witke et al., 1995). It has been shown that both gelsolin and capZ are inhibited in vitro by phospholipids of the D4 family such as P(4,5)P2 and P(3,4,5)P3 (Janmey and Stossel, 1989; Heiss and Cooper, 1991; Janmey,
1994). Although these lipids are initially hydrolysed by PLC upon platelet activation the levels rapidly rise, resulting in a 40% increase in phospholipids over the resting value. This de novo increase in lipids parallels the increase in the F-actin content in the cells. Using permeabilised platelets, it has been shown that thrombin receptor ligation stimulates the uncapping of actin filament barbed ends by ras-mediated stimulation of phosphoinositide synthesis (Hartwig et al., 1995). Introduction of peptides that mimic the phospholipid-binding site of platelet gelsolin inhibits ras and thrombin induced mobilisation of actin. Moreover such polyphosphoinositol lipids also impair the actin-binding ability of profilin, the actin monomer proteins thought to shuttle the monomer to the growing filament. A specific PIP2-binding site on profilin has recently been identified (Sohn et al., 1995). An attractive aspect of polyphosphoinositol lipids playing such a fundamental role in regulating actin polymerisation is that they can be produced at specific locations in the cell, potentially allowing them to co-ordinate actin mobilisation and polymerisation in a tightly regulated manner.

**Regulation of actin polymerisation in fibroblasts**

The situation described above introduces the mechanisms thought to regulate actin polymerisation within the platelet. In terms of actin polymerisation and filament formation in fibroblasts the details of regulation may differ. In motile fibroblasts the actin monomer addition has been shown to occur under the membrane, in a manner that is inhibited by the addition of capZ, implying that the barbed ends are uncapped in this situation (Barkalow and Hartwig, 1995). In migrating cells the regulation of actin polymerisation at the leading edge may well be similar to that observed upon the spreading or activation of a platelet, with localised generation of new F-actin filament meshwork in the leading edge, which is disassembled from the rear as the cell moves along (Stossel, 1993). Indeed a spreading platelet generates filopodia and lamellapodia as does a migrating fibroblast. This is supported in part by the observation that fibroblasts overexpressing gelsolin show increased motility, perhaps as a result of an increased capacity for rapid actin polymerisation (Cunningham et al., 1991). In terms of the system considered in this study the generation of an actin filament may well be slightly different. Actin stress fibres are assembled from the plasma membrane, the point at which they are anchored into the focal adhesion. One of the proteins present in the focal adhesion is an actin-capping protein tensin. Tensin is a weak actin capping protein and allows insertion of actin monomers into the filaments it caps (Lo et al., 1994a; Lo et al., 1994b). Therefore presentation of activated monomers to tensin would result in slow filament growth. Whether stress fibre formation results from *de novo* formation of F-actin or from the elongation of activated nucleation centres is not known. Talin has been reported to be an actin nucleating protein *in vitro*, and also augments F-actin bundling by α-actinin (Muguruma et al., 1990; Kaufmann et al., 1991; Kaufmann et al., 1992; Muguruma et al., 1992). A number of other actin binding proteins are localised to adhesions. Two of these proteins α-actinin and
vinculin also bind and are activated by polyphosphoinositol lipids (Fukami et al., 1992; Fukami et al., 1993; Weekes et al., 1996). A third protein VASP may also be important in this regard. VASP is a 46/50 kDa proline-rich serine phosphoprotein which decorates actin filaments and focal adhesions and is known to associate with zyxin (Haffner et al., 1995). By virtue of the homology it shares with the Listeria monocytogenes protein actA, VASP may play a role in co-ordinating the shuttling of actin monomers to the membrane for addition to the barbed end of the filament. When Listeria infect cells the bacteria move through cells and into neighbouring cells by propelling themselves on a "comet" of F-actin (Pistor et al., 1994; Southwick and Purich, 1994). The actA gene product, a bacterially expressed membrane protein, is solely responsible for the regulation of this actin polymerisation though it doesn't directly bind actin. The actA polyproline region inhibits this actin polymerisation when microinjected into infected cells. Interestingly vinculin and VASP also contains a similar proline rich sequence.

1.10 An overview of factors regulating fibroblast adhesion

The previous sections have discussed in isolation the various proteins involved in the regulation and structure of focal adhesions. In this section a brief synopsis of the factors known to regulate fibroblasts adhesions will be presented.

The formation of focal adhesions in fibroblasts has been studied in two ways; spreading cells on ECM components such as fibronectin, and stimulation of serum-starved cells with soluble extracellular factors, such as LPA and bombesin in serum-starved cells (Burridge et al., 1992; Ridley and Hall, 1992). Stimulation with members of both groups induces the tyrosine phosphorylation of a very restricted repertoire of proteins including tensin, pp125Fak, pp130cas, pp110AFAP, p80/85 cortactin and paxillin (Bockliot and Burridge, 1993; Burridge et al., 1992; Barry and Critchley, 1994, Ridley and Hall, 1994; Nojima et al., 1995; Vuori and Rouslahti, 1995). This tyrosine phosphorylation correlates with the formation of focal adhesions and actin stress fibres. When serum-starved mouse fibroblasts are stimulated with soluble factors, the tyrosine phosphorylation of these proteins appears dependent upon the small GTP-binding protein rho, as is the formation of focal adhesions and actin stress fibres (Rankin et al., 1994; Seekl et al., 1995; Ridley and Hall, 1992). Other members of the rho family of small GTP-binding proteins, rac and cdc42 regulate the formation of membrane ruffles and filopodia, respectively, in a sequential and co-ordinated manner, cdc42 stimulating rac, and rac stimulating rhoA. The formation of focal adhesions and actin stress fibres is also dependent on the action of tyrosine kinases. Spreading of fibroblasts on fibronectin can be inhibited by the tyrosine kinase inhibitor herbimycin A (Burridge et al., 1992), while the LPA-mediated formation of stress fibres and focal adhesions is inhibited by genistein and tyrphostin 25 (Ridley and Hall, 1992; Nobes et al., 1995). Moreover the formation of stress fibres and focal adhesions can also be driven by inhibiting tyrosine phosphatases (Barry and Critchley,
However the relationship between the activity of tyrosine phosphatases and kinase in the assembly and disassembly of focal adhesions is not known. The identity of tyrosine kinases involved in these signalling events are also unknown but two strong candidates are pp125Fak and pp60src.

Little is known of the mechanisms regulating the recruitment of β1-integrins and structural proteins such as vinculin, talin and α-actinin to the focal adhesion, or how actin is mobilised to form stress fibres. None of the structural proteins are tyrosine phosphorylated under conditions of adhesion formation (Bockholt and Burridge, 1993; Barry and Critchley, 1994) but may be recruited to the focal adhesion by phosphorylation on serine/threonine residues (Werth et al., 1983; Werth and Pastan, 1984; Lichfield and Ball, 1990a,b), or by interaction with acidic phospholipids which affect the activity of these proteins in vitro (Fukami et al., 1992; Weekes et al., 1996). Evidence suggests that actin may also be mobilised by the action of acidic phospholipids (Aderem, 1992; Sohn et al., 1995), though the regulation of actin in the adhesive response has not been assessed in detail. With the demonstration that a number of lipid modifying enzymes are regulated in vitro by rho it seems likely that lipids play an important role in regulating the formation of the focal adhesion (Zhang et al., 1993; Chong et al., 1994; Malcolm et al., 1994; Tolias et al., 1995). Early work on fibroblast adhesion implied that PKC activity is required for cells to spread on fibronectin (Woods and Couchman, 1992), and by inference from work in other cell lines PKC may be required for integrins to engage ECM components with high affinity (Vuori and Rouslahti, 1993). Again the role of PKC in fibroblast adhesion has not been studied in detail.
1.11 Aims of the project

Little is known of the mechanisms responsible for assembling the focal adhesion in vivo, although the in vitro properties of numerous cytoskeletal proteins have been studied in detail. Serum-starvation of Swiss 3T3 fibroblasts results in the loss of actin stress fibres and focal adhesions. The reformation of these structures is rapidly stimulated by the addition of LPA or FCS, in a rho-dependent manner (Ridley and Hall, 1992). Studying the assembly of focal adhesions in serum-starved Swiss 3T3 cells enables recruitment of proteins to nascent focal adhesions to be studied in detail, and correlated with changes in phosphorylation of specific proteins. The role of tyrosine kinases and tyrosine phosphatases in adhesion regulation will be defined using a variety of pharmacological agents, coupled with in vitro immune-complex enzyme assays.

Peptides corresponding to the cell-binding motif of fibronectin will be employed to assess the interaction made between serum-starved cells and the ECM in the absence of detectable focal adhesions and actin stress fibres. It is also possible to determine whether rho is also required for the integrin-mediated formation of actin stress fibres, and the tyrosine phosphorylation of proteins in serum-starved cells spread on fibronectin. Introducing C3 transferase into serum-starved cells enables the rho-dependency of this signalling event to be assessed.

The role of PKC in regulating the recruitment of certain cytoskeletal proteins to the focal adhesion will be addressed in serum-starved Swiss 3T3 cells. Using a pharmacological approach the effects of activating and inhibiting PKC will be assessed, along with the localisation of various PKC isoforms to focal adhesions. Finally a number of the structural focal adhesion proteins have been reported to be substrates for PKC. One such protein is vinculin (Werth et al., 1983; Werth and Pastan, 1984). The serine/threonine phosphorylation associated with recruitment of vinculin to the cytoskeleton will be determined upon stimulation of serum-starved Swiss 3T3 cells.
Chapter Two

Materials and Methods
2.1 General reagents and antibodies

2.1.1 General reagents

Unless otherwise stated all chemical reagents were of analytical grade, and most were obtained from BDH Laboratory Supplies (Lutterworth, Leicestershire, UK), Fisons (Loughborough, Leicestershire, UK) or Sigma Chemical Company Ltd, (Poole, Dorset, UK). Reagents for bacterial cell culture were obtained from Oxoid (Unipath, Basingstoke, Hampshire, UK). Reagents for manipulation of DNA were purchased from either Pharmacia Biotech (Milton Keynes, Buckinghamshire, UK.) or New England BioLabs (NEB) (c/o CP Labs, Bishops Stortford, Hertfordshire, UK).

2.1.2 Antibodies

Two monoclonal anti-phosphotyrosine antibodies were used. The first was obtained from Sigma (clone PT-66) and used at 1:5000-1:10 000 for Western blot analysis and at 1:1000 for immunofluorescence. Phosphotyrosine-containing proteins were immunoprecipitated with the same antibody conjugated to agarose beads (Sigma). The second monoclonal anti-phosphotyrosine antibody was obtained from Transduction Labornatories (Affiniti Research Products, Nottingham, U.K.) and used for Western blotting at 1:5000. A polyclonal anti-β1-integrin antibody (diluted 1:20 for immunoprecipitation) and an affinity-purified polyclonal anti-β1-integrin antibody (diluted 1:2000 for Western blotting) were obtained from Chemicon, Cricklewood, London, UK (product numbers AB1938 and AB1938P respectively). A mouse monoclonal anti-human vinculin antibody was a generous gift from Dr. V.E. Koteliansky (CNRS-Ecole Normale Superieure, Laboratoire de Physiopathology du Developpment, 46, rue D'Ulm, Paris, France) and was used for Western blot analysis at a dilution of 1:200, for immunofluorescence at 1:50, and for immunoprecipitation at 1:10. The second mouse anti-vinculin antibody, V284 was also used at 1:5000 for Western blotting and was a kind gift from Mike Wilkinson (Welcoome Trust, Euston Road, London). The mouse monoclonal anti-paxillin antibody was a kind gift from Dr. Chris Turner (SUNY Syracuse, USA) and used at 1:50 for immunofluorescence and immunoprecipitation, and at 1:150 for Western blot analysis. The polyclonal rabbit anti-talin antibody was a generous gift from Dr. K. Burridge (University of North Carolina, Chapel Hill, North Carolina, USA) and used at 1:500 for immunofluorescence. A monoclonal anti-chick pp125Fak antibody was a generous gift from Dr. T. Parsons (Health Sciences Centre, University of Virginia, Virginia USA) and was used at 1:50 for immunofluorescence, at 1:500 for Western blot analysis and 1:25 for immunoprecipitation. The monoclonal antibody to PKCθ was obtained from Transduction Laboratories (Affiniti Research Products, Nottingham, U.K.) and used as per manufacturers instructions. The rabbit polyclonal antibody to chicken gizzard smooth muscle α-actinin was produced in this laboratory and was used at 1:1000 for immunofluorescence. The monoclonal antibody 9E10 against the myc epitope tag was purified from the hybridoma originally described by Dr. G. Evan (Evan et al., 1985). All
agarose-conjugated products were purchased from Sigma. Horseradish peroxidase conjugated secondary antibodies for Western blotting were obtained initially from Amersham and in later experiments from Sigma.

2.2 Animal cell culture methods
2.2.1 Culture of Swiss 3T3 mouse fibroblasts
Swiss 3T3 mouse embryo fibroblasts used in all experiments were obtained from Dr. Anne Ridley (Institute of Cancer Research, London). They were routinely cultured in Dulbecco's Modified Eagle Medium (DMEM) (Gibco-BRL, Paisley, Glasgow, Scotland), supplemented with antibiotics and antimitotics (Gibco-BRL) and with 10% (batch tested) foetal calf serum (FCS) (Advanced Protein Products, Brierly Hill, U.K.). Cells were split when they had reached a maximum of 80% confluency. For immunofluorescent studies cells were grown on glass coverslips in 10 mm diameter 4 well tissue culture dishes, and for protein analysis in 90 mm tissue culture dishes. For serum-starvation experiments, cells were cultured to between 90 and 100% confluency over a period of 5 to 8 days. Cells were washed once in pre-warmed serum-free DMEM and then incubated for a further 16 hours in the serum-free medium (Ridley and Hall, 1992). Cells were used for no longer than 13 passages once thawed from frozen stocks. All tissue culture plastic was obtained from Nunc Products (supplied Gibco-BRL).

2.2.2 Culture of Balb/c 3T3 mouse fibroblasts
Balb/c 3T3 mouse embryo fibroblasts (ATCC No. CRL 1658) were routinely cultured in Dulbecco's Modified Eagle Medium (DMEM) (Gibco-BRL), supplemented with antibiotics and antimitotics (Gibco-BRL) and with 10% neo-natal calf serum (NCS) (Advanced Protein Products). For immunofluorescent studies, cells were grown on glass coverslips in 10 mm diameter 4 well tissue culture dishes and for protein analysis in 90mm tissue culture dishes. Cells were split at a maximum of 80% confluency. For serum-starvation experiments, cells were cultured to between 90 and 100% confluency over a period of 5 to 8 days. Cells were washed once in prewarmed serum-free DMEM, and then incubated for a further 1 to 2 hours in the serum-free medium.

Balb/c 3T3 fibroblasts transformed with the pCDNA-3 or pMAM-neo vectors were routinely cultured in media containing 50 μg/ml neomycin (G418) to ensure maintenance of each vector.

2.2.3 Culture of MRC5 human lung fibroblasts
MRC5 human embryo lung fibroblasts (ATCC No. CCL 171) were routinely cultured in DMEM without sodium pyruvate (Gibco-BRL) and supplemented with 10% NCS. For serum starvation cells were grown to 90-100% confluence over 5-8 days, washed once in serum-free DMEM (without sodium pyruvate) and then incubated for upto 24 hours in
2.2.4 Culture of chick embryo fibroblasts

The chick embryo fibroblasts used were obtained from 9 day gestation chick embryos and maintained as frozen stocks in liquid nitrogen. The head, limbs and gut of 9-10 day old chick embryos were removed and the remaining tissue finely minced. Following digestion with 0.25% trypsin initially at 4°C and then at 37°C (for 20 minutes each), the tissue fragments were pelleted and the supernatant filtered through sterile gauze to remove any large fragments. The resultant cells were pelleted, washed and plated in tissue culture dishes. The medium was changed after 2-3 hours to remove any contaminating myoblasts.

Cells were maintained in DMEM supplemented with antibiotics and anti-mycotics, 5% NCS, 1% chick serum (Gibco-BRL) and 10% tryptose phosphate (Gibco). For serum starvation, cells were grown to 90-100% confluence over 5-8 days, washed once in serum-free DMEM (without sodium pyruvate) and then incubated for up to 24 hours in serum-free DMEM.

2.2.5 Stimulation of serum-starved cells

All cells were stimulated with either 0.2% FCS, 100 ng/ml lysophosphatidic acid (LPA) (Sigma, Poole, Dorset), 50 ng/ml bombesin (Sigma) or 100 or 200 nM 12-0-tetradecanoylphorbol-13-acetate (TPA) (Sigma) for the times indicated. LPA was dissolved in sterile water at a concentration of 1 mg/ml and stored at -20°C for periods of up to 3 months prior to use. Bombesin was likewise dissolved in sterile water and stored at a stock concentration of 1 mg/ml at -20°C. TPA was dissolved at a stock concentration of 1.6 mM in dimethyl sulphoxide (DMSO) (Fluka) and stored at -20°C prior to use. As a result all control cells were treated with the relevant amount of DMSO or other appropriate solvent as described for each individual experiment.

2.2.6 Treatment of serum-starved cells with inhibitors

Cells were treated with a wide variety of kinase and phosphatase inhibitors. For all inhibitors dissolved in DMSO control cells were treated with the appropriate concentration of DMSO alone as indicated for each individual experiment. Cells were treated with the tyrosine kinase inhibitor genistein (Calbiochem, Nottingham U.K.) (Akiyama et al., 1987). Genistein was dissolved in DMSO at a stock concentration of 5 or 10 mg/ml and the cells treated with concentrations ranging from 2 to 30 μg/ml for 30 to 60 minutes prior to stimulation with 100 ng/ml LPA or spreading on fibronectin as described. Herbimycin A (Sigma) an alternative inhibitor of tyrosine kinases was also used (Uehara et al., 1989; Fukazawa et al., 1990). This light sensitive compound was dissolved in DMSO at a stock concentration of 174 μM in the dark and stored at -20°C prior to use. It was added to cells at a concentration of 174 nM for 16 hours throughout the starvation period essentially as
previously described by Burridge et al. (1992). The compound was protected from light as much as was reasonably possible.

To inhibit tyrosine phosphatases, cells were treated with and equimolar mix of H₂O₂ and sodium orthovanadate (as a 1 mM stock), which forms the cell-permeable tyrosine phosphatase inhibitor vanadyl hydroperoxide (orthovanadate) (Heffetz et al., 1990; Heffetz et al., 1992). Control cells were treated with H₂O₂ alone. A second more specific tyrosine phosphatase inhibitor, phenylarsine oxide (PAO), was also used to inhibit tyrosine phosphatase activity (Garcia-Morales et al., 1990). This was stored at room temperature as a stock of 10 mM in DMSO and diluted as appropriate immediately prior to use.

To inhibit protein kinase C, two different compounds were employed, both supplied by Calbiochem. Calphostin C was dissolved at a stock concentration of 1 mM in DMSO, and stored in the dark at -20°C until required. Calphostin C was added to the cells at a range of concentrations from 0.25-2.0 µM in the presence of a 7 Watt fluorescence light to light activate the compound (Kobayashi et al., 1989; Bruns et al., 1991). Bisindolylmaleimide was dissolved in DMSO at a stock concentration of 5 mg/ml and stored at -20°C until required (Toullec et al., 1991).

2.3 Immunological methods and gel electrophoresis

2.3.1 Immunofluorescence localisation of cytoskeletal proteins

Cells were generally washed quickly with PBS (twice) and fixed for 15 minutes in 3.7% (v/v) formaldehyde in PBS prior to immunostaining. The fixed cells were then washed with PBS and permeabilized with 0.2% Triton X-100 in PBS for 2 minutes. In the case of α-actinin immunostaining, cells were extracted with MES buffer (50 mM MES pH 6.0, 3 mM EGTA, 5 mM MgCl₂, 0.5% Triton X-100) for 2 minutes at 4°C prior to fixation to reduce cytoplasmic staining. To stain cells for PKCα, cells were methanol fixed. The cell were washed once in PBS and then incubated for 5 minutes at -20°C in 50% methanol, 50% acetic acid. Fixed cells were washed in PBS with numerous changes and then incubated with the primary antibody as described below.

Permeabilised cells were incubated at 37°C for 40 minutes with the appropriate primary antibody, washed with multiple changes of PBS, and stained with a 1:50 dilution of a TRITC-conjugated anti-mouse or anti-rabbit secondary antibody (Amersham, Aylesbury, UK). Cells were extensively washed with PBS and the distribution of F-actin detected by incubation (20 minutes) with fluorescein-conjugated phalloidin (Sigma) (diluted 1:100 in PBS). Cells were washed twice in PBS, the coverslips mounted on glass slides in 90% (v/v) glycerol, and examined using a Zeiss Axiophot epifluorescence microscope. Photographs were taken on Ilford HP5 Plus film (ASA 400) uprated to ASA 1600 during development.
2.3.2 Separation of proteins via SDS-polyacrylamide gel electrophoresis (SDS-PAGE).

Either a large Biorad gel electrophoresis or a LKB midget electrophoretic unit was used as a support to cast the acrylamide gels which were prepared as detailed in Table 2.1. The resolving gels, either 6, 7, 8, or 10%, and the stacking gels were prepared using a stock 30% acrylamide solution and polymerisation was initiated by addition of freshly prepared ammonium persulphate (Amm. Pers.) and TEMED. Samples were boiled into sample buffer (50 mM Tris, pH 6.8, 10% glycerol, 2% SDS, 0.1% bromophenol blue, 10% 2-mercaptoethanol, 1 mM sodium orthovanadate) for 2-5 minutes prior to loading on the gel. Gels were run in running buffer, 2.5 mM Tris, 19.2 mM glycine and 0.01% SDS, pH 8.3. Proteins were visualised by staining with 1% Coomassie Brilliant Blue (Sigma) made up in 10% acetic acid, 10% methanol, 80% H₂O. Excess stain was removed by extensive washing in the stain solution detailed above without Coomassie Brilliant Blue stain (Laemmli, 1970).

Table 2.1. SDS-polyacrylamide gels (all values in ml).

<table>
<thead>
<tr>
<th>Percentage gel Reagent</th>
<th>6% Lge.</th>
<th>6% Sml.</th>
<th>7% Lge.</th>
<th>7% Sml.</th>
<th>8% Lge.</th>
<th>8% Sml.</th>
<th>10% Lge.</th>
<th>10% Sml.</th>
<th>Stacking gel</th>
</tr>
</thead>
<tbody>
<tr>
<td>1M Tris pH 8.8</td>
<td>16.8</td>
<td>3.36</td>
<td>16.8</td>
<td>3.36</td>
<td>16.8</td>
<td>3.36</td>
<td>16.8</td>
<td>3.36</td>
<td>2.5</td>
</tr>
<tr>
<td>30% Acrylamide</td>
<td>8.8</td>
<td>1.94</td>
<td>10.2</td>
<td>2.13</td>
<td>11.7</td>
<td>2.33</td>
<td>13.1</td>
<td>2.92</td>
<td>2.7</td>
</tr>
<tr>
<td>Water</td>
<td>19.0</td>
<td>3.38</td>
<td>17.6</td>
<td>3.18</td>
<td>16.0</td>
<td>2.98</td>
<td>14.5</td>
<td>2.4</td>
<td>14.7</td>
</tr>
<tr>
<td>20% SDS</td>
<td>0.225</td>
<td>0.045</td>
<td>0.225</td>
<td>0.045</td>
<td>0.225</td>
<td>0.045</td>
<td>0.22</td>
<td>0.045</td>
<td>0.100</td>
</tr>
<tr>
<td>10% Amm. Pers.</td>
<td>0.150</td>
<td>0.2</td>
<td>0.150</td>
<td>0.2</td>
<td>0.150</td>
<td>0.2</td>
<td>0.150</td>
<td>0.2</td>
<td>0.100</td>
</tr>
<tr>
<td>TEMED</td>
<td>0.03</td>
<td>0.01</td>
<td>0.03</td>
<td>0.01</td>
<td>0.03</td>
<td>0.01</td>
<td>0.03</td>
<td>0.01</td>
<td>0.03</td>
</tr>
</tbody>
</table>

All volumes are shown in ml. Lge represents volumes for a large gel and Sml volumes for a small gel.

2.3.3 Western blot analysis

Immune precipitates were routinely boiled in loading buffer prior to separation of the proteins by SDS-PAGE. For Western analysis the separated proteins were transferred to Hybond-C extra. Cell monolayers in 9 cm dishes were washed once with PBS (4°C) and whole cell lysates prepared by scraping the contents of the dish into 200μl of protein loading buffer. Lysates were boiled immediately (5 minutes) and stored at -20°C. The proteins present in equal volumes of cell lysates were resolved in 8% SDS-polyacrylamide gels and the proteins electroblotted to Hybond-C Extra nitrocellulose (Amersham). Large gels were transferred for 2 hours 30 minutes and small gels for 45-60 minutes at 200 mA.
Transfer buffer consisted of 1.5 mM Tris (not pH'd), 200 mM glycine and 10% methanol.

Blots were stained with Ponceau-S to confirm that protein loading was equal in all lanes and to visualise the molecular weight markers. Filters were soaked in TBS (pH 7.5) to remove the Ponceau S, and incubated in TBS pH 7.5 containing 5% dried skimmed milk or BSA and 0.1% Tween 20 for 1 hour to block excess protein-binding sites. Filters were then incubated (1 hour) with the appropriate primary antibody diluted in the TBS pH 7.5 containing 1% dried skimmed milk or BSA and 0.1% Tween 20 followed by either anti-mouse or anti-rabbit antibodies conjugated to Horse Radish Peroxidase (Amersham diluted 1:5000; Sigma diluted 1:3000). The proteins were detected using an ECL chemiluminescence kit (Amersham) and the light emitted detected with Fuji Medical X-Ray Film. Where necessary, the primary and secondary antibodies were stripped from the filter as per manufacturer's instructions, and the filters reprobed with additional antibodies.

2.3.4 Immune precipitation.

For all immune precipitations, lysates corresponding to an entire 9 cm dish were used in each experiment. Cells from 9 cm dishes were scraped into 200 μl of RIPA buffer (0.01 M Tris pH 7.0, 0.15 M NaCl, 2 mM EDTA, 1 mM sodium orthovanadate, 0.1% SDS, 1% NP40, 1% sodium deoxycholate and 2 mM PMSF plus the protease inhibitors E64 (5 μg/ml) and leupeptin (5 μg/ml) at 4°C. The DNA was sheared by passage through a fine needle and cell debris removed by centrifugation at 13000 rpm for 15 minutes (4°C). To immune precipitate vinculin and β1-integrin, the lysate was pre-absorbed with 30 μl protein A or G coupled to agarose beads (Sigma) for 1 hour, and the beads removed by centrifugation. The primary antibody was then added (2 hours, 4°C) to the entire supernatant, and the immune complexes precipitated by incubating with protein A- or G-conjugated agarose beads for 2 hours at 4°C. The beads were pelleted, washed several times with NET buffer (0.05 M Tris pH 7.0, 150 mM NaCl, 5 mM EDTA, 0.1% BSA, 0.1% SDS, 0.1% sodium deoxycholate and 0.5% NP40) and boiled for 5 minutes in 100 μl of protein loading buffer. Equal volumes of the solubilised material were analysed in 8% SDS-polyacrylamide gels, and the proteins blotted to nitrocellulose. Filters were stained with Ponceau-S to ensure that equal levels of protein were present in all lanes. The success of the immune precipitation was confirmed by staining the filters for either vinculin or β1-integrin, and the presence of phosphotyrosine in these proteins investigated using an anti-phosphotyrosine antibody.

For immune precipitation of pp125Fak and paxillin, cells were lysed by scraping into ice cold NP40 lysis buffer (10 mM Tris pH 7.4, 5 mM EDTA, 50 mM NaCl, 30 mM sodium pyrophosphate 0.1 mM sodium orthovanadate, 1% Triton X-100, 1 mM PMSF). This was because neither of the respective antibodies could precipitate protein in the presence of SDS. The lysates were then passed through a hypodermic needle and clarified by spinning
at 13000 rpm at 4°C for 15 minutes. The clarified lysates was then precleared by incubation with albumin conjugated to agarose beads (Sigma) for 1 hour. The beads were pelleted and the supernatant incubated by rotation with either the anti-pp125Fak monoclonal 2A7 or the anti-paxillin monoclonal for 2 hours at 4°C. Agarose-anti mouse IgG or agarose-anti-mouse IgG1 (both Sigma) was then added for a further hour and the mixture spun at 6500 rpm to pellet the beads and proteins. The immune-precipitate was then washed 4 times in lysis buffer and boiled in protein loading buffer. The precipitates were then analysed by Western blotting with the anti-phosphotyrosine monoclonal. The blot was stripped as described above, and reprobed for the protein of interest to determine the quantity of the protein on the blot.

Phosphotyrosine-containing proteins were precipitated with an anti-phosphotyrosine antibody conjugated to agarose beads (Sigma). Lysate corresponding to an entire 9 cm dish was incubated with 15 μl of the beads for 3 hours at 4°C, the beads pelleted and washed three times in RIPA buffer. The phosphotyrosine-containing proteins were then eluted from the beads by boiling into protein loading buffer, and the samples were then analysed for the presence of pp125Fak and paxillin by Western blotting.

2.4 Preparation and use of adhesion motif peptides

2.4.1 Preparation of peptides for adhesion competition assays

The adhesion motif peptides were synthesised using fmoc chemistry on an ABI 431A synthesiser (kindly provided by Prof. Martin Humphries, University of Manchester). Peptides (20 mg/300 μl) were first desalted on a 10 ml G10 Sephadex column (Whatman) packed in, and eluted with 10 mM NH$_4$HCO$_3$. The void volume of the column containing the desalted peptides was collected and lyophilised. The peptides were then stored at -20°C, and dissolved immediately before use in serum-free DMEM at a stock concentration of 1 mg/ml.

2.4.2 Cell adhesion assays

For peptide competition assays, cells were cultured in 1 cm diameter multi-well dishes, serum starved as usual and treated with the appropriate amount of peptide in a total of 200 μl. Throughout the duration of the assay the cells were regularly inspected by phase microscopy.

Cells were stained with crystal violet as followed. The media was removed, the cell monolayer washed very carefully once in PBS and then fixed in 3.7% paraformaldehyde for 30 minutes. The cells were then washed carefully with PBS and the stained with 0.02% crystal violet in H$_2$O, for 15 minutes. Cells were rinsed once more in PBS and visualised by phase contrast using a Nikon TMS tissue culture microscope. Photographs were taken on Ilford HP5 Plus film (ASA 400).
2.5 Microinjection and scrape loading of C3 transferase

These methods were used by Drs. Anne Ridley and Helen Flinn (Ludwig Institute for Cancer Research, Riding House St., London) as part of a collaboration investigating the mechanisms of integrin-mediated signalling (Chapter 4).

2.5.1 Microinjection of C3 transferase

Recombinant C3 transferase was expressed in *E. coli* as a GST-fusion protein using the vector pGEX-2T, and purified as previously described (Ridley and Hall, 1992). Swiss 3T3 cells (3x10⁴) were seeded on 13 mm glass coverslips. After 5 to 7 days, cells were serum-starved in DMEM for 16 hrs. Approximately 100 cells were microinjected with 2 µg/ml C3 transferase and 0.5 mg/ml rat IgG as a marker protein. Cells were then incubated for 15 minutes at 37°C prior to addition of the peptides, to allow the C3 transferase to inhibit rho proteins. Cells were fixed at the times indicated, permeabilised and actin filaments localized with TRITC-conjugated phalloidin as previously described (Ridley and Hall, 1992).

2.5.2 Scrape-loading of cells with C3 transferase

Cells were seeded at a density of 6x10⁵ per 9 cm dish in 10% FCS in DMEM. After 3 days they were starved of serum for 16 hrs. Cells were scrape loaded as described previously (Morris et al., 1989; Levers and Marshall, 1992). Briefly, C3 transferase (230 µg/ml) in 160 µl of scrape loading buffer (10 mM Tris-HCl pH 7.0, 114 mM KCl, 15 mM NaCl, 5.5 mM MgCl₂) was added to the cell monolayer, and the cells detached from the dish using a rubber policeman. For immunofluorescence analysis cells from one 9 cm dish were plated in serum-free DMEM on 9 glass coverslips (13 mm diameter) coated with human fibronectin (Sigma) (50 µg/ml, 37°C, 90 minutes). After the times indicated, cells were stained with TRITC-phalloidin as described above. For Western blotting, cells from one 9 cm dish were plated on one fibronectin-coated dish, or held in suspension, and incubated at 37°C for 30 minutes.

2.6 *In vitro* kinase and phosphatase assays

2.6.1 pp125Fak kinase assay

For pp125Fak kinase assays, proteins were either immuneprecipitated with the anti-pp125Fak monoclonal or with agarose-anti-phosphotyrosine, as described above. The washed precipitates were then washed four times in kinase buffer (20 mM Tris pH 7.4, 150 mM NaCl, 1 mM MnCl₂, 1 mM MgCl₂, 100 µM Na₃VO₄) and resuspended in a total volume of 50 µl kinase buffer. The reaction was started by adding 5 µCi of γ³²P-ATP (specific activity 111 TBq/mMol (Amersham International, Little Chalfont, U.K.), and allowed to proceed for 25 minutes. The precipitate was then washed four times in a large volume of kinase buffer, boiled in protein loading buffer, and centrifuged for 5 minutes at
13000 rpm (Wilson et al., 1995). The phosphorylated products were resolved on an 8% polyacrylamide gel, the gel stained with Coomassie blue and the phosphorylated products visualised by autoradiography or phosphorimaging.

2.6.2 Protein kinase C kinase assay

To assay the ability of PKC to phosphorylate certain proteins, a brain preparation of mixed PKC isoforms (Calbiochem) was incubated with the putative substrate in the presence of phosphatidyl serine (Lipid Products S. Nutfield, U.K.) and the PKC activator TPA. Lipids were prepared by drying 1 mg phosphatidyl serine and 1 µg TPA to the side of a glass walled tube under a stream of nitrogen. The lipids and TPA were then redissolved in 1% Triton X-100, 20 mM Tris pH 7.5, at a concentration of 0.5 mg/ml phosphatidylserine, 0.5 µg/ml TPA by warming and sonicating with two 5 second pulses in a sonicating water bath. Lipids were stored at -20°C and used for up to 4 weeks. In each reaction substrate was added along with 10 mM MgCl₂, 6.7 mM CaCl₂ (as required), 0.175 mg/ml phosphatidyl serine, 0.175 µg/ml TPA, and 100 µM ³²P-ATP. An appropriate amount of enzyme, either PKC-δ alone, or a mix of PKC isoforms was added to each reaction. Each assay was started by adding either ATP or enzyme.

Reactions were terminated by adding protein loading buffer. The samples were then boiled, proteins resolved in an 8% gel, and stained with Coomassie blue. ³²P-labelled proteins were visualised by either autoradiography or by phosphorimaging.

2.6.3 Tyrosine phosphatase assay

Preparation of substrate

Substrate was prepared by treating normal 90% confluent cultured Swiss 3T3 fibroblasts cultured in 15 cm plates with the tyrosine phosphatase inhibitor PAO at 5 µM for 10 minutes in serum-free DMEM. The dishes were then washed twice in PBS and lysed into 700 µl NP40 lysis buffer as described above. pp125Fak and paxillin were immunoprecipitated from these lysates also as described above, except that the antibodies were incubated with the lysate overnight at 4°C. The resultant precipitate was extensively washed and finally washed into assay buffer (50 mM HEPES pH 7.4, 0.5% Triton X-100, 5 µg/ml leupeptin, 5 µg/ml E64). Equal volumes of the substrates bound to agarose beads were then added to individual assay tubes.

Phosphatase assay

The tyrosine phosphatase activity against both pp125Fak and paxillin in Swiss 3T3 cell lysates was assayed. Treated cells were likewise washed twice in PBS and then lysed into phosphatase assay lysis/assay buffer (50 mM HEPES pH 7.4, 0.5% Triton, 5 µg/ml leupeptin, 5 µg/ml E64). The lysate was cleared by spinning for 45 minutes at 13000 rpm (4°C), and the protein concentrations of each supernatant estimated with a Coomassie
protein analysis kit (Pierce, used as per manufacturers instruction). The phosphatase activity against pp125Fak and paxillin was then measured for each sample by incubating lysate with substrate for the appropriate length of time at 37°C with agitation to keep the beads in suspension. The reaction was finally stopped by putting the tubes on ice and adding stop solution (50 mM TBS, 0.5% Triton X-100, 0.1% SDS). Each reaction was boiled into protein loading buffer for subsequent separation by SDS-PAGE and analysis by Western blotting using the PY20 anti-phosphotyrosine antibody.

2.7 Analysis of phosphorylated proteins

2.7.1 Rehydration of dried gels and transfer of proteins onto PVDF membrane

Bands corresponding to $^{32}$P-labelled proteins were excised from dried gels and rehydrated by incubating in a large volume of H$_2$O overnight at room temperature. The rehydrated gel was then soaked in 1% SDS at room temperature for 1 hour, and then soaked in transfer buffer for 30 minutes to remove excess SDS. PVDF membrane (0.2 μm, obtained from Pall) was presoaked in methanol and then in PVDF transfer buffer (48 mM Tris, 39 mM glycine, 10% methanol, 0.03% SDS). Transfer was allowed to proceed for 2-3 hours at 200 mA, which transferred the majority of the protein from the gel.

2.7.2 Phosphoamino acid analysis

Proteins blotted onto PVDF were subjected to acid hydrolysis in 6M HCl for 1 hour at 110°C. The hydrolysate was lyophilised overnight to remove residual acid and then the membrane and hydrolysis tube washed in 200 μl H$_2$O containing 30 μg/ml phosphoamino acid standards. The membrane was then removed and the free amino acids re-lyophilised and resuspended in 10 μl H$_2$O. The solution was then spotted onto a TLC plate and subjected to flat bed electrophoresis in one dimension. This was carried out at 1 kV for 1 hour in water: acetic acid: pyridine (945:50:5, pH 3.5). Following separation the amino acids standards were visualised by autoradiography or by phosphorimaging.

2.7.3 Phosphopeptide analysis

Proteins blotted onto PVDF were digested overnight using 10 mg/ml CNBr and then the resulting peptides eluted from the membrane in peptide elution buffer (70% isopropanol, 0.002% trifluoroacetic acid, 0.001% lysine, 0.001% thioglycolic acid). Elution was carried out at room temperature over 2 days. The resulting eluent was then lyophilised and resuspended in protein gel loading buffer. The peptides were resolved on a 3 phase gel. (4% acrylamide stacking gel, 10% spacer gel, and a 16.5% separating gel containing 9% glycerol). The peptide gel buffer consisted of 0.66 M Tris pH 8.45 and 0.066% SDS. The anode buffer consisted of 0.2 M Tris pH 8.9 and the cathode buffer consisted of 0.1 M Tris, 0.1 M Tricine, 0.1% SDS pH 8.0-8.5. The gel was stained with Coomassie blue and the phosphopeptides were visualised by phosphorimaging.
2.8 Metabolic cell labelling and cell fractionation

2.8.1 \( ^{35}S\)-methionine cell labelling

To metabolically label cellular proteins with \( ^{35}S\)-methionine, cells were grown to approximately 80% confluency. The cells were then washed once with PBS, then with pre-warmed methionine-free DMEM (Gibco). Cells were incubated for 40 minutes in methionine free media supplemented with either 10% FCS or appropriate serum. The media was then removed and replaced with methionine-free DMEM supplemented with serum and either \( ^{35}S\)-methionine (Amersham) or a mix of \( ^{35}S\)-methionine-cysteine (DuPont NEN) at a concentration of 50 \( \mu Ci/ml. \) The cells were then incubated for a further 16-18 hours before either being lysed in the appropriate lysis buffer or being subjected to cell fractionation.

2.8.2 \( ^{32}P\)-orthophosphate labelling of Swiss 3T3 cells

To determine the degree of serine and threonine phosphorylation of certain proteins \textit{in vivo} cells were metabolically labelled with \( ^{32}P\)-orthophosphoric acid. Cells were either grown to confluency for serum-starvation experiments or to 80% confluency for labelling of growing cultured cells. Cells were washed twice in phosphate-free DMEM (ICN-flow) and then incubated for a further 45 minutes in phosphate-free DMEM. For serum-starvation experiments cells were then incubated for a further 16 hours in 5 ml serum-free DMEM supplemented with 100 \( \mu Ci/ml \) \( ^{32}P\)-orthophosphoric acid, carrier free (Amersham). For labelling growing cells in culture, the cells were incubated for 16 hours in phosphate-free DMEM supplemented with dialysed serum containing no inorganic phosphate and 100 \( \mu Ci/ml \) \( ^{32}P\)-orthophosphoric acid. Cells were harvested by washing 5 times in cold PBS to remove excess unincorporated phosphate and then either lysed or extracted as appropriate.

2.8.3 Preparation of Triton X-100 soluble and insoluble fractions

All fractionation experiments were performed on cells plated in 9 cm dishes. Serum-starved or serum-stimulated cells, and cells plated on fibronectin, were initially washed with cold PBS. To obtain the Triton soluble fraction, cells were washed with 800 \( \mu l \) Triton extraction buffer (0.2% or 1% Triton X-100, 10 mM Tris-HCl pH 7.6, 50 mM NaF, 1 mM sodium orthovanadate, 5 mM EDTA, 1 mM EGTA, 50 mM NaCl. 30 mM sodium pyrophosphate, 1 mM PMSF, 5 \( \mu g/ml \) E64, 5 \( \mu g/ml \) leupeptin) for 2 or 4 minutes as indicated. The Triton soluble fraction was then completely removed and the Triton X-100 insoluble cytoskeleton obtained by dissolving the remainder of the cells in 100 \( \mu l \) of a buffer containing 2% SDS (2% SDS, 1% Triton X-100, 1% sodium deoxycholate, 20 mM Tris-HCl pH 7.0, 50 mM NaF, 1 mM sodium orthovanadate, 1 mM EDTA, 150 mM NaCl, 1 mM PMSF, 5 \( \mu g/ml \) E64, 5 \( \mu g/ml \) leupeptin) and heating to 80°C for 4 minutes. This Triton X-100 insoluble fraction was then cooled on ice for 2 minutes and finally the SDS
content diluted by addition of diluent buffer (1% Triton X-100, 1% sodium deoxycholate, 20 mM Tris-HCl pH 7.0, 50 mM NaF, 1 mM sodium orthovanadate, 1 mM EDTA, 150 mM NaCl, 1 mM PMSF, 5 μg/ml E64, 5 μg/ml leupeptin).

To fractionate cells in suspension the cells were first pelleted by spinning at 13000 rpm at 4°C for 1 minute and then resuspended in the appropriate extraction buffer or SDS lysis buffer. To separate the Triton X-100 soluble and Triton X-100 insoluble fractions the cells were spun at 13000 rpm (4°C), after the appropriate incubation in the extraction buffer, which pelleted the Triton X-100 insoluble cytoskeleton. The soluble supernatant was removed and the insoluble fraction taken into the appropriate amount of SDS solubilisation buffer as described for adherent cells.

The concentrations of the Triton X-100, SDS and sodium deoxycholate were adjusted such that approximately equivalent concentrations of each detergent were present in each sample, before being subjected to immuneprecipitation.

2.9 Molecular biology techniques
2.9.1 Media recipes
Working stocks of E. coli strain JM101 were maintained on M9 minimal media plates (see below) and cultures of non-transformed JM101 were grown in 2TY media (see below) without ampicillin. Working stocks of E. coli strains JM109 were maintained on 2TY media plates without ampicillin and cultures of non-transformed JM109 were grown in 2TY media without ampicillin. Once bacteria had been transformed with plasmid DNA they were maintained on ampicillin-containing agar plates and all liquid cultures were grown in ampicillin-containing 2TY media as detailed below. Reserve stocks of all E. coli cultures were preserved in 20% glycerol/80% 2TY at -70°C. Following autoclaving of the different media ampicillin was added to a final concentration of 100 μg/ml where appropriate.

M9 Minimal Media plates were composed of the following ingredients: 6 g Na₂HPO₄, 3 g KH₂PO₄, 0.5 g NaCl, 1 g NH₄Cl, 15 g Bacto-Agar, 900 ml deionised water. Once autoclaved and cooled to approximately 50°C, the following was added: 1 ml 1 M MgSO₄, 10 ml 10 mM CaCl₂, 10 ml 20% glucose, 1 ml 10 mg/ml thiamine and deionised water to 1 litre.

2TY media was made up as follows: 16 g tryptone, 10 g yeast extract, 5 g NaCl and deionised water to 1 litre.

Standard agar plates were made up as follows: 10 g tryptone, 5 g yeast extract, 10 g NaCl, 1 g glucose, 15 g Bacto-agar and de-ionised water to 1 litre.
2.9.2 Small-scale plasmid preparation

Plasmid DNA from bacterial cultures of up to 5 ml was prepared using the Wizard miniprep DNA purification kit (Promega) as described in the manufacturer's instructions. Briefly, a cleared bacterial cell lysate was prepared by a standard alkaline lysis procedure, precipitated bacterial protein and chromosomal DNA pelleted by spinning for 15 minutes at 13000 rpm. The resultant cleared lysate was then combined with a suspension of silica-based resin which preferentially binds DNA. The DNA bound to this resin was washed and then eluted by subsequent rapid rehydration of the resin by the addition of water allowing plasmid DNA to be released.

2.9.3 Ethanol precipitation of DNA

DNA was precipitated by the addition of 3 M sodium acetate (pH 5.2) to 0.3 M followed by addition of 2 volumes of absolute ethanol. The sample was incubated at -20°C for 20 minutes and the DNA pelleted by centrifugation for 20 min at 13000 rpm at 4°C. The supernatant was removed and the DNA pellet washed with 70% ethanol and dried before being resuspended in sterile distilled water (Sambrook et al., 1989).

2.9.4 Phenol extraction

To remove contaminating proteins from solutions of DNA, an equal volume of phenol:chloroform (1:1) was added to the solution. The two phases were then mixed for 30 seconds and the aqueous and organic phases separated by centrifugation for 5 min at 13000 rpm in a microfuge. The aqueous top layer was carefully removed and transferred to a clean tube. DNA was then ethanol precipitated and resuspended in distilled sterile water.

2.9.5 Synthesis and purification of oligonucleotides

Oligonucleotides were synthesised on an Applied Biosystems model 394 machine (Protein and Nucleic Acid Sequencing Laboratory, Leicester University) at 0.2 μmol or 1 μmol scales. Oligonucleotides were purified from the by-products of synthesis by ethanol precipitation with 0.1 volumes of 3 M sodium acetate (pH 4.5) to a concentration of 0.3 M followed by the addition of 2 volumes of ethanol. After incubation at -20°C for 30 minutes, the precipitated DNA was pelleted by centrifugation at 13000 rpm for 20 minutes at 4°C. The pellet was then washed with 70% ethanol and the pellet resuspended in an equal volume of sterile distilled water. The concentration of the precipitated oligonucleotide was determined by measuring the absorbance of the sample at OD260. The absorbance of a 1 mM solution of each oligonucleotide was estimated using the following calculation; each individual A has an absorbance value of 15.4, each T a value of 8.8, each C a value of 7.3 and each G a value of 11.4.
2.9.6 Amplification of cDNA by the polymerase chain reaction.
Amplification of cDNA sequences by the polymerase chain reaction (PCR) was carried out essentially as described by Sambrook et al., (1989). The concentration of each oligo was determined by an OD$_{260}$ reading, and then diluted to a working concentration of 10 µM. Reactions were generally carried out using approximately 5 ng-10 ng of plasmid DNA in 1x PCR buffer (50 mM KCl, 10 mM Tris-HCl pH 8.0, 1.5 mM MgCl$_2$), with 200 µM of each dNTP (Promega) and 25 pmol of each oligonucleotide primer. All annealling temperatures were set at 5°C below that of the melting temperature for each individual primer. The melting temperature was estimated by assigning a melting value of 2°C for every A/T and 4°C for every G/C. All PCR reactions were carried out in a Biometra 30 Thermoblock.

For subsequent subcloning following PCR amplification, an aliquot of the reaction was analysed by agarose gel electrophoresis, and the remainder of the reaction subjected to phenol/chloroform extraction and ethanol precipitation for restriction digest. The DNA pellet was resuspended in sterile distilled water.

2.9.7 Preparation of DNA for ligations and ligation reactions
Vector DNA was prepared using a Promega Wizard or Magic miniprep column for the purification of small amounts DNA and used as per manufacturers instructions. The eluted DNA was then subjected to ethanol precipitation and resuspended in distilled sterile water. Appropriate restriction enzyme sites were incorporated into the 5' ends of the oligonucleotide primers for amplification of cDNA inserts by PCR. 1-5 µg of both vector DNA and amplified cDNA was then subjected to digestion with appropriate restriction enzyme. Cut DNA was then purified by agarose gel electrophoresis, the band excised and the DNA extracted using a Qiagen Gel Extraction kit as per manufacturers instructions. An aliquot of the purified DNA fragments was then analysed on a small agarose gel to estimate the relative concentrations of each DNA solution.

All ligations were carried out in standard 1x New England Biolab (NEB) ligase buffer using NEB DNA ligase (1 enzyme unit per reaction) overnight at 16°C in a total volume of either 10 or 20 µl. Insert cDNA was present at approximately a ten fold excess over vector DNA in each ligation reaction.

2.9.8 Preparation of competent E.coli for transformation
The following protocol was used for the production of competent JM101 and JM109. A single colony of cells were grown overnight in 2TY without ampicillin. The next morning the culture was back-diluted 1:100 in fresh 2TY medium (10 ml) and grown for a further 2 hours. The bacterial cells were then pelleted and resuspended in 10 ml of ice-cold 50 mM CaCl$_2$, re-pelleted and resuspended in 0.5 ml 50 mM CaCl$_2$. Cells were then either left to
stand on ice for several hours before use or stored at 4°C overnight for use the following day. Immediately prior to addition of DNA 1 μl of DMSO was added to 0.5 ml of resuspended cells. 100 μl of cells were used for each transformation.

2.9.9 Transformation of competent cells
Half the ligation reaction was added to 100 μl of competent cells and incubated on ice for 30 minutes. Following a heat-shock step, at 37°C for 2 minutes for JM101 and JM109 cells, and 42°C for 2 minutes for MC1016 cells, the cells were incubated on ice for a further 10 minutes. 8 volumes of 2TY medium was added and the cells grown at 37°C for 90-120 minutes with occasional agitation. The cells were then pelleted at 6500 rpm in a microfuge, spread onto agar plates and incubated overnight at 37°C to allow the ampicillin resistant bacterial colonies to grow.

2.9.10 Analysis of putative transformants by PCR
Initial screening for putative positive transformants was carried out by PCR analysis of a crude preparation of bacterial DNA. Colonies resulting from each transformation were picked into 3 ml of 2TY media containing 100 μg/ml ampicillin and grown for 5-6 hours. 500 μl of the culture was then taken, the bacteria pelleted and resuspended in 200 μl distilled sterile water. The suspension was then boiled for 10 minutes to lyse bacteria and the insoluble material pelleted by centrifugation for 10 minutes at 13000 rpm. 5 μl of the supernatant containing the liberated bacterial and vector DNA was then removed and subjected to PCR amplification using the oligonucleotide primers initially used to amplify the cDNA insert of interest as described above. Positive clones identified in this manner were then further investigated by purification of plasmid DNA followed by diagnostic restriction endonuclease digestion.

2.9.11 DNA sequencing
Sequencing of plasmid DNA was performed using CircumVent thermal cycle dideoxy DNA chain termination sequencing (New England Biolabs) (Sanger et al., 1977). DNA for sequencing was prepared using DNA purification columns (Promega) as described earlier but prior to sequencing plasmid DNA was further purified by PEG precipitation. This was achieved by adding 0.4 M NaCl and 6.5% PEG8000 to the miniprep DNA followed by an incubation on ice for 20 min. Plasmid DNA was then pelleted by centrifugation at 13000 rpm for 15 min at 4°C in a fixed-angle rotor. The resultant pellet was washed with 70% ethanol, air dried and resuspended in water. An aliquot of the DNA was then run on a small agarose gel to determine the approximate concentration of the DNA. The sequencing reactions were performed using the the Circumvent sequencing kit as per manufacturers instructions. All annealing temperatures were set at 5°C below that of the melting temperature for each individual primer. 35S-dATP was obtained from Amersham. The sequencing products were analysed on denaturing 6% polyacrylamide
gels (acrylamide:bis, 19:1) containing 8 M urea.

**2.9.12 Oligonucleotide primers**
The following oligonucleotides were used to generate various PCR products shown in the 5’ to 3’ orientation.

<table>
<thead>
<tr>
<th>Oligo (against)</th>
<th>Sequence (restriction enzyme sites underlined)</th>
</tr>
</thead>
<tbody>
<tr>
<td>STB3 (5' PKCÔ cDNA)</td>
<td>AAA GGA TCC GCA CCG TTC CTG CGC A</td>
</tr>
<tr>
<td>STB4 (3' PKCÔ cDNA)</td>
<td>AAA GAA TTC TTC CAG GAA TTG CTC ATA TT</td>
</tr>
<tr>
<td>STB8 (3' PKCÔ cDNA)</td>
<td>CTA GTC TAG ACT ATT CCA GGA ATT GCT CAT ATT</td>
</tr>
<tr>
<td>STB9 (5' myc tag)</td>
<td>GCC GCT CGA GAT GGA GCA GAA GCT GAT C</td>
</tr>
<tr>
<td>PKCd-1 (5' PKCÔ cDNA)</td>
<td>GGG CTT CTT CTG TAC CAG</td>
</tr>
</tbody>
</table>

**2.10 Analysis of PKCδ localisation**

**2.10.1 Preparation of pMT-PKCδ DNA for transfection into COS cells**
pMT-PKCδ construct obtained from Dr. Peter Parker (ICRF, London) was transformed into JM109 competent cells. pMT is a high copy number mammalian expression vector used for transient transfection of mammalian cells. The PKCδ sequence included in the plasmid contained both coding sequence and 5’ and 3’ untranslated sequence. Plasmid DNA was isolated from a 200 ml culture using a Promega DNA maxiprep spin column as per manufacturers instructions and concentrated by ethanol precipitation.

**2.10.2 Transient transfection of monkey Cos cells**
Cos cells were maintained in DMEM supplemented with 10% NCS. Cells for transformation were plated at 3x10^5 per 9 cm dish. For fluorescence analysis, 2 cm glass coverslips were included in the dish. The cells were allowed to settle and spread overnight then incubated in serum-free DMEM for 5 minutes, then in 4 ml of 50 mM Tris pH 7.5 with between 5 and 20 µg/ml DNA and 1 ml DEAE dextran (at 1 mg/ml) in Tris Buffered Saline (140 mM NaCl, 0.75 mM Na₂HPO₄, and 2.5 mM HEPES pH 7.1) and incubated for 6-8 hrs at 37°C. The media was then removed and the cells were shocked in 10% DMSO Hepes Buffered Saline for 4 minutes, the solution removed and the cells washed with serum-free DMEM. The cells were then cultured for 36-40 hrs in DMEM with 10% NCS, before being examined for expression of PKC-δ by immunofluorescence or Western blotting.

**2.10.3 Construction of myc epitope tagged expression constructs**
To determine the cellular localisation of PKCδ, the cDNA corresponding to the full coding sequence of the protein was epitope tagged at the N-terminus with a myc epitope recognised by the monoclonal antibody 9E10 (Evan et al., 1985; Kanai et al., 1993).
To generate the myc-tagged protein, the PKCδ coding sequence was first amplified by PCR using primers designed to the 5' (STB3) and 3' (STB4) ends of the sequence excluding the initiating ATG encoding the initiating methionine. The primers had a BamHI and EcoRI site incorporated at the 5' and 3' ends respectively. The vector containing the coding sequence for the myc epitope was originally constructed in the laboratory of Richard Triesman (ICRF Lincolns Inn Fields, London) and is essentially the pGEM2 vector with the residues corresponding to the epitope tag inserted 5' to the multiple cloning site. Both the PCR fragment and the vector were cut with BamHI and EcoRI gel purified, ligated and transformed into JM101 as described above. The resultant myc-PKCδ fusion was then sequenced with a primer directed against the 5' end of the PKC sequence (PKCd-1), complementary to the sense strand, to ensure that the myc tag DNA sequence was in frame with that encoding the PKC-δ.

For subsequent expression in mammalian cells this fusion was then inserted into the mammalian expression vectors pCDNA3 (Invitrogen, San Diego, USA) and pMAMneo-BLUE (Clontech, Paolo Alto, USA). pCDNA3 allows constitutive expression of a protein in stably transformed cells while pMAMneo-BLUE allows inducible expression of protein under the control of the MMTV promoter, expression being induced by the corticosteroid dexamethasome. A DNA fragment consisting of the myc-PKCδ fusion was again generated by PCR with primers directed against the 5' end of the myc epitope (STB9) and the 3' end of the PKCδ sequence (STB8). The 5' primer also introduced an XhoI cloning site into the fragment prior to the first residue of the myc epitope, while the 3' primer introduced a XbaI site after the 3' end of the PKCδ sequence. The PCR generated DNA fragment and each vector (pCDNA3 and pMAMneo-BLUE) were cut to completion with XhoI and XbaI, gel purified, ligated and transformed into competent MC1061, as described above. The myc-PKCδ pCDNA3/pMAMneo-BLUE constructs were again sequenced with the primer PKCd-1 to ensure that the reading frame was maintained from the myc epitope through to the PKCδ sequence.

2.10.4 Stable transfection of Balb/c 3T3 with PKCδ expression constructs

Balb/c 3T3 fibroblasts were transformed using the CaPO₄ method of transfection. Cells were initially seeded at a density of 5x10⁵ cells per 9 cm dish 24 hours prior to transfection. Approximately 20 μg of each plasmid DNA was diluted in 2 mls of 1x Hepes buffered saline (HBS) (140 mM NaCl, 0.75 mM Na₂HPO₄, 2.5 mM HEPES) containing 125 mM CaCl₂, and air was bubbled through the mixture for 30 seconds to induce the formation of a precipitate. Following a 30 minute incubation at room temperature, 1 ml of the DNA/CaCl₂ mixture was added to each of two dishes and returned to the incubator for a further 6 hours. The medium was then removed from the plates and the cells rinsed twice in 5 mls PBS. Each dish was then incubated in 1 ml of 20% glycerol:PBS for 90
seconds and then washed again twice in 5 ml PBS. Fresh media containing 7 mM sodium butyrate and 10% NCS was then added to the cells and the dishes returned to the incubator for a further 12 hours. The cells were then washed again in DMEM containing 10% NCS and incubated in media containing serum for a further 36 hours. Each dish of cells was then trypsinised and split into five 9 cm dishes with media containing 0.5 mg/ml G418 (Gibco, Life Technologies) for selection of positive clones. The G418 containing growth media was replaced every 3-4 days until isolated colonies of resistant cells were seen. Each clone was picked from the parent dish by tower cloning. Essentially the dishes were rinsed twice in PBS, and a small plastic cylinder, dipped in vaseline to provide a watertight seal, placed over each individual colony. 200-300 µl of trypsin were pipetted into each cylinder, and once cells had completely detached 200-300 µl of medium added to each cylinder. The cells were then removed to a 3 cm dish containing fresh medium plus 0.5 mg/ml G418. Each clone was expanded as appropriate into 9 cm dishes at which point the cells were trypsinised and resuspended in a mixture of 65% FCS, 25% DMEM and 10% DMSO for long term storage in liquid nitrogen. Clones were cultured in the presence of G418 to maintain the plasmid. To induce expression of protein from the pMAMneo-BLUE vector the cells were incubated in the presence of 1 µM dexamethasone (Sigma, stock concentration of 100 µM dissolved in ethanol) for 4-5 days with the dexamethasone being replenished every day. Clones of pCDNA3- mycδ and pMAM-mycδ were then assayed for expression of the myc tagged PKCδ by Western blotting with the 9E10 anti-myc monoclonal antibody as ascites fluid diluted at 1:400.
The 2.06 kb myc tagged PKC-δ DNA was generated by PCR and cloned into the XhoI XbaI sites of the pcDNA3 plasmid. The maintenance of the reading frame between the myc tag and PKC was confirmed by dideoxy-chain termination sequencing.
The 2.06 kb myc tagged PKC-δ DNA was generated by PCR and cloned into the Xho I site of the pMAMneoBlue plasmid. The reading frame of the myc-PKC was confirmed by dideoxy chain termination sequencing. SV40 sequences provide the polyA adenylation sites for correct processing of cloned insert and the promoter for neomycin resistance gene.
Chapter Three

Formation of focal adhesions and actin stress fibres in mouse fibroblasts
3.1 Introduction
A number of model systems that enable the signalling mechanisms associated with cell-matrix adhesion to be studied have been described. In fibroblasts, the formation of focal adhesions and associated actin stress fibres can be stimulated either by ECM components such as fibronectin via integrins (Burridge et al., 1992), or by soluble mitogens via a pathway dependent on the small GTP-binding protein rhoA (Ridley and Hall, 1992). Burridge and co-workers (1992) first demonstrated that spreading of a variety of fibroblast cell lines on fibronectin stimulated the tyrosine phosphorylation of a specific array of proteins of molecular weights 210, 130, 125, 110, 80, and 66 kDa, and a time dependent formation of focal adhesions and actin stress fibres. Study of the mitogen-stimulated rhoA-dependent formation of focal adhesions has utilised a system in which mouse Swiss 3T3 fibroblasts are deprived of serum for 16 hours, resulting in almost complete loss of both focal adhesions and associated actin stress fibres. Reformation of focal adhesions and stress fibres can be stimulated by addition of 0.2% FCS to the media in a manner that is inhibited by micro-injection of the rho inhibitor C3 transferase. This response can be mimicked by the addition of the bioactive lipid lysophosphatidic acid (LPA) or the neuropeptide bombesin to the serum-free media (Ridley and Hall, 1992). This system has been utilised to further investigate events regulating the formation of focal adhesions and actin stress fibres. The chapter is divided into two parts, the first describing the characterisation of the system, and the second describing the role that tyrosine phosphatases play in regulating the assembly of focal adhesions and actin stress fibres.

3.2 Results Part 1: Characterisation of the assembly of focal adhesions in serum-starved Swiss 3T3 cells

3.2.1 Serum-starved Swiss 3T3 cells assemble focal adhesions in response to addition of serum
As originally described by Ridley and Hall (1992), removal of serum from Swiss 3T3 fibroblasts results in a loss of actin stress fibres and vinculin-containing focal adhesions (Fig. 3.1A,B). This situation is rapidly reversed by readdition of low levels (0.2%) of FCS to the media (Fig. 3.1C,D). Other cytoskeletal proteins such as talin (Fig. 3.1E-H) and paxillin (Fig 3.2A-D) are also recruited to focal adhesions localised to the ends of actin stress fibres, while the actin-bundling protein α-actinin decorates the actin stress fibres emanating from the focal adhesions (Fig. 3.1I-L). The focal adhesion associated tyrosine kinase pp125Fak is recruited to these newly formed adhesions (Fig. 3.2E-F), and indeed staining with an antibody against phosphotyrosine shows that the focal adhesions stain heavily for phosphotyrosine-containing proteins (Fig. 3.2 I-L). The serum-starved cells frequently display a characteristic punctate staining pattern for actin throughout the cytoplasm as well as plasma membrane staining (for example see Fig. 3.1E). None of the
focal adhesion associated proteins could be visualised by immunofluorescence in serum-starved cells, even in cells that contained residual actin stress fibres. (Figs. 3.1E and 3.2A).

3.2.2 Time course of serum-induced formation of actin stress fibres and focal adhesions

To assess the speed with which focal adhesions and actin stress fibres are assembled, serum-starved cells were fixed 2 minutes, 5 minutes and 10 minutes after the addition of 0.2% FCS, and the cells double stained for actin and either paxillin (Fig. 3.3), vinculin (Fig. 3.4) or with an anti-phosphotyrosine antibody (Fig. 3.5).

Serum-starved cells again displayed few actin stress fibres and no paxillin-containing focal adhesions (Fig. 3.3A,B). Within 2 minutes of the addition of serum there was strong punctate paxillin staining around the periphery of the cell (Fig. 3.3D), although the actin filaments were only marginally more pronounced than those displayed by the serum-starved cells. In some cases, paxillin was localised to the ends of fine actin filaments (Fig. 3.3C,D). Within 5 minutes the actin filaments became more clearly defined (Fig. 3.3E) with paxillin localised to the ends of each filament (Fig. 3.3F). The focal structures stained ranged from circular to oval, but by 10 minutes had become more elongated (Fig. 3.3G,H) and closely resembled the focal adhesions found in Swiss 3T3 grown under standard conditions.

Analysis of the recruitment of vinculin to newly forming adhesions revealed that there was no detectable difference in the rate of recruitment compared with paxillin. No vinculin-containing focal adhesions were visible in serum-starved cells (Fig. 3.4B). However clusters of vinculin, associated with faint actin fibres could be detected only 2 minutes after addition of serum (Fig. 3.4C,D). More distinct focal clusters of vinculin could be seen 5 minutes after the addition of serum, at which point newly formed actin filaments were clearly visible associated with these clusters (Fig. 3.4E,F). After 10 minutes the vinculin-containing adhesions began to more closely resemble adhesions found in cultured fibroblasts (Fig. 3.4 G,H).

When cells were double stained for actin and phosphotyrosine a slightly different pattern of staining was obtained. As before, serum-starved cells displayed punctate staining for actin and very little distinct staining for phosphotyrosine (Fig. 3.5A,B). However, 2 minutes after the addition of serum, the cells exhibited punctate staining for phosphotyrosine distributed both around the periphery of the cell, and within the body of the cell. Whilst the staining at the periphery was clearly associated with the ends of very fine actin filaments, the punctate staining in the body of the cell appeared to correspond with the distribution of the punctate actin (Fig. 3.5C,D). After 5 minutes of serum treatment the staining became more focal, and localised to the periphery of the cell at the
ends of fine actin filaments (Fig. 3.5E,F). There was little obvious change in the pattern of phosphotyrosine distribution when cells were incubated for 10 minutes (Fig. 3.5G,H).

These results indicate that the formation of focal adhesions in response to serum is a rapid and progressive event. Structural proteins rapidly form focal complexes in a manner that correlates closely with the stimulation of tyrosine phosphorylation of components of the adhesive structures.

3.2.3 Lysophosphatidic acid-induced formation of focal adhesions

The bioactive lipid lysophosphatidic acid has been shown to induce the formation of actin stress fibres in serum-starved Swiss 3T3 cells (Ridley and Hall, 1992). To assess whether the effects of LPA treatment were the same as that of FCS-stimulation, serum-starved cells were treated with 100 ng/ml LPA for 20 minutes. LPA triggered both the formation of actin stress fibres and the recruitment of vinculin, paxillin and pp125Fak to newly formed focal adhesions within 20 minutes. These newly formed adhesions also stained strongly for phosphotyrosine-containing proteins (Fig. 3.6).

3.2.4 Serum and LPA induce tyrosine phosphorylation of the same repertoire of proteins

To compare the effects of both serum and LPA on the tyrosine phosphorylation of cellular proteins, serum-starved cells were treated with 0.2% FCS or 100 ng/ml LPA for 10 and 20 minutes. Equal amounts of whole cell extracts were separated by SDS-PAGE and the tyrosine phosphorylation of cellular proteins analysed by Western blotting with an anti-phosphotyrosine antibody. Both serum and LPA induced a marked increase in tyrosine phosphorylation of proteins of around 116 kDa and of a diffuse doublet around 60-70 kDa (Fig. 3.7). Increased tyrosine phosphorylation could also be detected in a protein of around 80 kDa. Two of these proteins are of a similar molecular weight to proteins that have previously been shown to exhibit increased tyrosine phosphorylation when cells spread on fibronectin, namely paxillin (68 kDa) and the focal adhesion kinase pp125Fak (125 kDa) (Burridge et al., 1992). These results are consistent in at least six similar experiments. This suggests that signalling elements are common to both the LPA/rhoA- and fibronectin/integrin-mediated pathways responsible for stimulating the formation of focal adhesions and associated actin stress fibres.

3.2.5 pp125Fak and paxillin both exhibit increases in tyrosine phosphorylation in response to serum and LPA-stimulation

To analyse whether pp125Fak and paxillin were indeed two of the proteins undergoing changes in tyrosine phosphorylation during serum- and LPA-induced formation of focal adhesions, serum-starved and stimulated cells were lysed and the tyrosine phosphorylated proteins precipitated with an anti-phosphotyrosine antibody conjugated to agarose beads. The immuneprecipitate was then analysed by SDS-PAGE and Western blotting with
Figure 3.1. Serum-induced formation of actin filaments and focal adhesions in Swiss 3T3 cells. Localisation of vinculin, talin and α-actinin. Semi-confluent Swiss 3T3 cells were cultured in serum-free medium for 16 hrs. Some of the cells were then fixed and stained for F-actin (A,E,I) and double stained for either vinculin (B), talin (F), or α-actinin (J). Other cells were treated with fresh medium containing 0.2% foetal calf serum for 20 min and fixed and stained for F-actin (C,G,K) with double staining for vinculin (D), talin (H), and α-actinin (L). Magnification bar 5μm.
Figure 3.2. Serum-induced formation of actin filaments and focal adhesions in Swiss 3T3 cells. Localisation of paxillin, pp125FAK, and phosphotyrosine-containing components. Semi-confluent Swiss 3T3 cells were cultured in serum-free medium for 16 hrs. Some of the cells were then fixed and stained for F-actin (A,E,I) and double stained for either paxillin (B), pp125FAK (F), or phosphotyrosine (J). Other cells were treated with fresh medium containing 0.2% foetal calf serum for 20 min and fixed and stained for F-actin (C,G,K) with double staining for paxillin (D), pp125FAK (H), and phosphotyrosine (L). Magnification bar 5μm.
Figure 3.3. Time course of the formation of actin filaments and paxillin-containing focal adhesions. Serum-starved Swiss 3T3 cells were treated with 0.2% FCS for 0 min (A,B), 2 min (C,D), 5 min (E,F) or 10 min (G,H) and the cells fixed and double stained for F-actin (A,C,E,G) and paxillin (B,D,F,H). Magnification bar 5μm.
Figure 3.4. Time course of the formation of actin filaments and vinculin-containing focal adhesions. Serum-starved Swiss 3T3 cells were treated with 0.2% FCS for 0 min (A,B), 2 min (C,D), 5 min (E,F) or 10 min (G,H) and the cells fixed and double stained for F-actin (A,C,E,G) and vinculin (B,D,F,H). Magnification bar 5μm.
Figure 3.5. Time course of the formation of actin filaments and phosphotyrosine-containing focal adhesions. Serum-starved Swiss 3T3 cells were treated with 0.2% FCS for 0 min (A,B), 2 min (C,D), 5 min (E,F) or 10 min (G,H) and the cells fixed and double stained for F-actin (A,C,E,G) and phosphotyrosine (B,D,F,H). Magnification bar 5µm.
Figure 3.6. Lysophosphatidic acid-induced formation of actin filaments and focal adhesions in Swiss 3T3 cells. Swiss 3T3 cells cultured in serum-free medium for 16 hrs were fixed and stained for F-actin (A,E,I,M) and double stained for either vinculin (B), paxillin (F), pp125FAK (J) and phosphotyrosine (N). Other cells were treated with fresh medium containing 100 ng/ml lysophosphatidic acid for 20 min and fixed and stained for F-actin (C,G,K,O) with double staining for vinculin (D), paxillin (H), pp125FAK (L) and phosphotyrosine (P). Magnification bar 5μm.
antibodies to pp125Fak and paxillin. The amounts of pp125Fak (Fig. 3.8A) and paxillin (Fig. 3.8B) markedly increased in cells treated with both serum and LPA. The simplest interpretation of this result is that both pp125Fak and paxillin show an increase of tyrosine phosphorylation upon stimulation of serum-starved Swiss 3T3 with serum or LPA. This was consistent in a number of similar experiments. These experiments do not however formally exclude the possibility that pp125Fak and paxillin immuneprecipitate with the anti-phosphotyrosine antibody because of an association with another protein that is itself tyrosine phosphorylated.

To address this possibility, pp125Fak and paxillin were directly immuneprecipitated from lysates of serum-starved cells and cells stimulated with LPA, the immuneprecipitate resolved by SDS-PAGE and proteins detected by Western blotting with an anti-phosphotyrosine antibody. (Figs. 3.9A and 3.10A respectively). Both pp125Fak and paxillin from stimulated cells showed elevated phosphotyrosine content. The blots were then stripped and reprobed with antibodies to both pp125Fak and paxillin to visualise the amount of precipitated protein present on each. Although there appears to be slightly less pp125Fak (Fig. 3.9B) precipitated from the serum-starved lysate compared with the LPA-treated lysate, this does not account for the difference seen with respect to the amount of tyrosine phosphorylated protein isolated from each lysate. This observation was subsequently confirmed by Ridley and Hall, (1994), who also observed that pp125Fak exhibited increased tyrosine phosphorylation during LPA-stimulation of serum-starved Swiss 3T3. The amounts of paxillin present in both the serum-starved and LPA-stimulated lysates are equivalent (Fig. 3.10B). These observations are representative of at least two similar experiments concerning both pp125Fak and paxillin. The mobility shift of paxillin evident in Fig. 3.10B arises from multiple phosphorylation of paxillin, not only on tyrosine but also serine/threonine residues (Turner and Miller, 1994; Salgia et al., 1995). The results confirm that both pp125Fak and paxillin are tyrosine phosphorylated cytoskeletal proteins that exhibit increased tyrosine phosphorylation in response to stimulation of serum-starved Swiss 3T3 cells with LPA.

3.2.6 Stimulation of serum-starved Swiss 3T3 with the neuropeptide bombesin
Similar changes in tyrosine phosphorylation can also be stimulated by treating the cells with the neuropeptide bombesin. Serum-starved cells stimulated with 50 ng/ml bombesin exhibit a marked increase in tyrosine phosphorylation of proteins of molecular weight 60-70 kDa, 80 kDa and 116 kDa, evident as early as 5 minutes after stimulation (Fig. 3.11A). Bombesin also induced the formation of actin stress fibres and phosphotyrosine- and vinculin-containing focal adhesions (data not shown).

Analysis of the phosphotyrosine immuneprecipitate from bombesin-stimulated cells revealed that both pp125Fak and paxillin show increased tyrosine phosphorylation upon
treatment of cells with bombesin (Fig. 3.12A,B). The results show that another extracellular factor, bombesin, acts through a signalling pathway similar to that of serum/LPA to exert effects on cellular adhesion. The responses are also similar to those generated when fibroblasts are allowed to spread on fibronectin (Burridge et al., 1992).

3.2.7 Tyrosine phosphorylation of specific proteins occurs rapidly in response to LPA-stimulation of serum-starved Swiss 3T3

Although we have demonstrated that the changes in tyrosine phosphorylation are detectable as quickly as 2 minutes after stimulation with serum by fluorescence, it is not possible to analyse any earlier time point using this technique. To assess how quickly LPA induces tyrosine phosphorylation of specific cellular proteins, serum-starved cells were stimulated with 100 ng/ml LPA for 30 seconds and 2 minutes, the media removed and the cells immediately lysed into SDS-loading buffer. Equal amounts of lysate were separated by SDS-PAGE and analysed by Western blotting with an anti-phosphotyrosine antibody. A clear increase in tyrosine phosphorylation of proteins of 60-70 kDa and 116 kDa can be seen within 30 seconds of the addition of LPA to the cells (Fig. 3.13).

When a second anti-phosphotyrosine antibody (PY20), was used instead of PT66, it became apparent that PT66 did not recognise all tyrosine phosphorylated residues present on proteins phosphorylated in response to LPA. Serum-starved Swiss 3T3 were stimulated for either 30 seconds, 1, 2, and 5 minutes or 30 seconds, 2, and 5 minutes with LPA and the tyrosine phosphorylation of the individual proteins analysed (Fig. 3.14). This revealed that additional proteins, most importantly at 110 and 130 kDa, become rapidly tyrosine phosphorylated in response to LPA. This can be seen as a triplet of proteins migrating around the 116 kDa marker, instead of the single band seen when lysates are Western blotted with PT66. The diffuse complex of bands migrating just above the 66 kDa marker probably corresponds to paxillin, with a similar band seen when lysates are Western blotted with PT66. The results show that anti-phosphotyrosine antibodies may not recognise all the phosphotyrosine residues present within proteins.

To confirm that the increase in tyrosine phosphorylation of pp125Fak and paxillin paralleled this increase in total cellular tyrosine phosphorylation, serum-starved cells were stimulated with LPA for 30 seconds, 2 minutes and 20 minutes, the media removed and the cells immediately lysed. The lysates were then subjected to immunoprecipitation with anti-phosphotyrosine conjugated agarose beads. The immunoprecipitate was analysed by Western blotting with antibodies to both pp125Fak and paxillin (Fig. 3.15A,B). A marked increase in the anti-phosphotyrosine precipitable pp125Fak and paxillin occurs after only 30 seconds of treatment with 100 ng/ml LPA. The signalling events associated with the formation of focal adhesions and actin stress fibres therefore occurs extremely rapidly after stimulation of serum-starved cells with LPA. This rapid increase in the tyrosine
phosphorylation of specific proteins correlates with the observation that focal complexes begin to appear as early as 2 minutes after stimulation.

3.2.8 \(\beta_1\)-integrin and vinculin do not undergo tyrosine phosphorylation in response to serum-stimulation

Two other focal adhesion associated proteins, \(\beta_1\)-integrin and vinculin, also co-migrate at approximately 116 kDa and could also therefore contribute to the tyrosine phosphorylation observed at this position on Western blots. Indeed vinculin is known to undergo tyrosine phosphorylation in fibroblasts transformed with the v-src oncogene (Kellie et al., 1986). To determine whether these proteins contribute to the tyrosine phosphorylated band at 116 kDa, vinculin and \(\beta_1\)-integrin were directly immunoprecipitated from lysates of serum-starved and serum-stimulated cells. The immunoprecipitate was then analysed by Western blotting with an anti-phosphotyrosine antibody (Fig. 3.16A,B). Neither protein appeared to be tyrosine phosphorylated under the conditions of assay. Stripping and reprobing with antibodies against vinculin and \(\beta_1\)-integrin revealed that both had been precipitated with equal efficiency from each lysate (Fig. 3.16A,B). These observations were consistent between two independent experiments analysing both \(\beta_1\)-integrin and vinculin. Immunoprecipitation with the \(\beta_1\)-integrin antibody should precipitate any associated \(\alpha\)-subunits, although this has not been confirmed directly by Western blotting. Assuming this to be the case, there is no evidence for tyrosine phosphorylation of associated \(\alpha\)-subunits.

3.2.9 Serum-starvation and stimulation of other fibroblast cell lines

It is possible that the signalling events associated with adhesion formation in serum-starved Swiss 3T3 may be restricted to one individual cell line. To address this question, a variety of fibroblast cell lines were assessed for the ability to exhibit a similar response to serum-starvation and subsequent serum-stimulation. The following cell types were studied:

Chick Embryo Fibroblasts (CEF) - Cells were deprived of serum for 24 hours and then stimulated with 0.2% of the normal culture serum. The serum-deprived cells did not appear to exhibit a decrease in the level of protein tyrosine phosphorylation when analysed by Western blotting, nor in the number and distribution of focal adhesions by immunofluorescence (data not shown). However there did appear to be a change in the pattern of actin staining upon serum-starvation. Serum-starved cells appeared to possess thick actin cables, while the serum-stimulated cells stained more intensely with phalloidin, and contained some fine actin filaments.

MRC5 human lung fibroblasts - Again cells were serum-starved for 24 hours and then stimulated by addition of 0.2% FCS to the media. Serum-starved cells contained thick actin cables which terminated in large focal adhesions while the serum-stimulated cells showed a
Figure 3.7. Serum and lysophosphatidic acid-induced tyrosine phosphorylation in Swiss 3T3 cells. Swiss 3T3 cells cultured in serum-free medium for 16 hrs were exposed to fresh medium either containing 0.2% FCS or 100 ng/ml lysophosphatidic acid (LPA) for 0 min, 10 min and 20 min. Panel A; the proteins present in equal amounts of cell lysates were resolved by SDS-PAGE, blotted to nitrocellulose and the blot stained with antibodies to phosphotyrosine (PT66). Molecular weight markers (kDa) are represented on the left hand side of the blot.

Figure 3.8. Paxillin and pp125Fak undergo increased tyrosine phosphorylation upon stimulation of serum-starved Swiss 3T3 with FCS and LPA. The phosphotyrosine-containing proteins were precipitated from equal amounts of cell lysates with an anti-phosphotyrosine antibody PT66 coupled to agarose. Lysates were made from either serum-starved or cells stimulated with 0.2% FCS or 100 ng/ml LPA for 10 and 20 minutes. Precipitated proteins were dissolved in SDS-sample buffer, resolved by SDS-PAGE and blotted to nitrocellulose. The blots were stained with an antibody to pp125FAK (Panel A) or paxillin (Panel B). Molecular weight markers (kDa) are shown to the left of each panel.
Figure 3.9. **Tyrosine phosphorylation of pp125Fak in response to LPA stimulation of serum starved Swiss 3T3.** pp125Fak was directly immuneprecipitated from lysates of serum-starved cells (0) and cells stimulated for 20 minutes with 10 ng/ml LPA (20). The precipitated protein was dissolved in protein loading buffer, resolved by SDS-PAGE, Western blotted with an anti-phosphotyrosine antibody PT66 (panel A), and then stripped and reprobed with an anti-pp125Fak antibody (panel B) to determine the pp125Fak in each lane. Molecular weight markers (kDa) are indicated on the left of each panel.

Figure 3.10. **Tyrosine phosphorylation of paxillin in response to LPA stimulation of serum-starved Swiss 3T3 cells.** Paxillin was directly immuneprecipitated from lysates of serum-starved cells (0) and cells stimulated for 20 minutes with 10 ng/ml LPA (20). The precipitated protein was dissolved in protein loading buffer, resolved by SDS-PAGE, Western blotted with an anti-phosphotyrosine antibody PT66 (panel A), and then stripped and reprobed with an anti-paxillin antibody (panel B) to determine the pp125Fak in each lane. Paxillin is visible at approximately 66 kDa and the precipitating immunoglobulins at 50 kDa. Molecular weight markers (kDa) are indicated on the left of each panel.
Figure 3.11. *Stimulation of serum-starved Swiss 3T3 cells with the neuropeptide bombesin.* Serum-starved cells (0) were stimulated with either 50 ng/ml bombesin for 5 minutes (5), and 20 minutes (20), or 100 ng/ml LPA for 20 minutes (LPA (20)). The cells were lysed, boiled into protein loading buffer and equal amounts of lysate resolved by SDS-PAGE. The gel was then analysed by Western blotting with an anti-phosphotyrosine antibody PT66 (panel A) and then stripped and reprobed with an anti-vinculin antibody (panel B) as a measure of the protein present in each lane of the blot. Molecular weight markers (kDa) are represented on the left of each panel.

Figure 3.12. *Bombesin stimulates tyrosine phosphorylation of pp125Fak and paxillin.* Serum-starved Swiss 3T3 cells (0) were stimulated with 50 ng/ml bombesin for 20 minutes (20), lysed and the tyrosine phosphorylated proteins precipitated from the entire lysate with an anti-phosphotyrosine antibody (PT66) conjugated to agarose beads. The resulting phosphotyrosine fractions were resolved by SDS-PAGE and the presence of both pp125Fak (panel A) and paxillin (panel B) detected by Western blotting with antibodies against the respective proteins. Molecular weight markers (kDa) are represented on the left of each panel.
Figure 3.13. Early time points of LPA stimulation of serum-starved Swiss 3T3. Serum-starved Swiss 3T3 (0) were stimulated with 100 ng/ml LPA for 30 seconds (30") and 2 minutes (2'). Equal numbers of cells were scraped into protein loading buffer, equal amounts of lysate resolved by SDS-PAGE and Western blotted with an anti-phosphotyrosine antibody PT66 (panel A). The blot was then stripped and reprobed with an anti-vinculin antibody as a control for equal loading (panel B). Molecular weight markers (kDa) are represented on the left of each panel.
Figure 3.14. Western blotting of LPA-stimulated Swiss 3T3 with the anti-phosphotyrosine antibody PY20. Western blotting with a second anti-phosphotyrosine antibody revealed that other proteins also increased tyrosine phosphorylation upon LPA stimulation. Serum-starved Swiss 3T3 cells (0) were stimulated for 30 seconds (30''), 1 minute (1'), 2 minutes (2') and 5 minutes (5'). An independent set of serum-starved Swiss 3T3 cells (0) were also stimulated for 30 seconds (30''), 2 minutes (2') and 5 minutes (5') with 100 ng/ml LPA. Equal amounts of cell lysate were resolved on two gels and one Western blotted with the anti-phosphotyrosine antibody PY20 (panel A) and the other Western blotted with an antibody against vinculin (panel B) to determine the protein present in each lane. Molecular weight markers (kDa) are represented on the left hand side of each panel.
Figure 3.15. pp125Fak and paxillin are rapidly tyrosine phosphorylated in response to LPA. Serum-starved cells (0) were stimulated with 100 ng/ml LPA for 30 seconds (30''), 2 minutes (2') or 20 minutes (20'). The phosphotyrosine containing proteins were then immuneprecipitated from the entire lysate and the precipitate resolved by SDS-PAGE and Western blotted with antibodies to pp125Fak (panel A) and paxillin (panel B). Molecular weight markers (kDa) are represented on the left hand side of each panel.
Figure 3.16. Analysis of the effect of serum on the tyrosine phosphorylation of vinculin and β1-integrins. Vinculin (A) and β1-integrins (B) were immune precipitated from equal numbers of serum-starved cells (0 min) and serum-stimulated cells (20 min) as described in materials and methods. Immune precipitated proteins were resolved by SDS-PAGE and blotted to nitrocellulose. The blots were probed with an anti-phosphotyrosine antibody, then stripped and reprobed with the antibody against the protein of interest, namely vinculin (panel A) and β1-integrin (panel B). Molecular weight markers (kDa) are shown to the left of each panel.
redistribution of this actin into finer filaments and also staining of actin distributed under
the membrane. This change in actin distribution can be easily detected by fluorescence,
with the cell membrane staining brightly with phalloidin. Serum-stimulation of these cells
also led to an increase in the number of, but a decrease in the size of the focal adhesions
associated with the finer actin filaments. Although this cell line is not useful as an assay
system for focal adhesion formation, the observation do confirm that serum-dependent
reorganisation of the actin cytoskeleton and associated adhesive structures does occur in
other cell lines.

RAT1 rat fibroblasts - Again the fibroblasts were grown to confluence and then
subsequently serum starved for 24 hours prior to stimulation with 0.2% FCS. When
examined by fluorescence, the serum-starved cells appeared to possess fewer actin stress
fibres and associated focal adhesions when compared to serum-stimulated cells. However
when changes in the tyrosine phosphorylation of cellular proteins were analysed, no
difference could be detected between the starved and stimulated state. Subsequently a
number of reports in the literature have demonstrated that RAT1 fibroblasts do indeed
exhibit the same responses to serum-deprivation and stimulation as Swiss 3T3 cells
(Hordijk et al., 1994; Saville et al., 1994). This suggests that the cell line held in the lab was
spontaneously transformed, a theory borne out by the observation that the cells showed
little or no contact inhibition as they reached confluence, typical of a transformed fibroblast
cell line (data not shown).

Balb/c 3T3 mouse fibroblasts - This cell line has been used previously to study the effects
of serum-deprivation on the onset of apoptosis (Tamm et al., 1992). One observation that
this group made was that upon serum withdrawal, the cells exhibited a loss of actin stress
fibres within 1-2 hours. The events associated with the formation of focal adhesions and
actin stress fibres in Balb/c 3T3 fibroblasts were therefore analysed for comparison with
those observed in the Swiss 3T3 cell system.

3.2.10 Serum-starved Balb/c 3T3 cells show a marked increase in tyrosine
phosphorylation upon serum-stimulation

Confluent Balb/c 3T3 cells were serum-starved for either 30 minutes or 2 hours, then
stimulated with 0.2% FCS for 20 minutes. Equal amounts of cell lysate were separated by
SDS-PAGE and analysed for tyrosine phosphorylated proteins by Western blotting with
an anti-phosphotyrosine antibody (Fig. 3.17). Cells starved for both 30 minutes and 2
hours exhibited a basal level of tyrosine phosphorylation of specific proteins which was
increased upon the addition of 0.2% FCS to the media. The profile of tyrosine
phosphorylated proteins observed was identical to that seen in Swiss 3T3 cells, and was
consistent in a number of independent experiments. Although Balb/c 3T3 fibroblasts will
serum-starve in as little as 30 minutes, for further experiments a starvation period of 1 hour 45 minutes was used.

A protein of approximately 210 kDa also exhibits changes in tyrosine phosphorylation upon stimulation of Balb/c 3T3 cells. A 210 kDa protein known as tensin has been shown to undergo tyrosine phosphorylation when cells spread on fibronectin and when fibroblasts are stimulated with FCS or LPA (Bockholt et al., 1992; Bockholt and Burridge, 1993). The identity of the protein could not be confirmed as an antibody against tensin that reacts with mouse protein was not available. This 210 kDa tyrosine phosphorylated protein is usually not visible on blots of whole cell lysates because its high molecular weight means it does not transfer well under standard conditions.

3.2.11 Serum-stimulation of serum-starved Balb/c 3T3 cells leads to the formation of actin stress fibres and focal adhesions

90% confluent Balb/c 3T3 cells were serum-starved for approximately 1 hour 45 minutes and then stimulated for 20 minutes with 0.2% FCS. Serum-starved cells also exhibited the punctate actin staining characteristic of serum-starved Swiss 3T3 cells and contained little or no actin filaments (Fig. 3.18A,E,I) or focal adhesions as visualised by staining for phosphotyrosine (Fig. 3.18B), paxillin (Fig. 3.18F) or vinculin (Fig. 3.18J). Stimulation with 0.2% FCS led to the formation of actin stress fibres (Fig. 3.18C,G,K) and focal adhesions staining for phosphotyrosine (Fig. 3.18D), paxillin (Fig. 3.18H) and vinculin (Fig. 3.18L).

3.2.12 Both FCS- and LPA-stimulation of serum-starved Balb/c 3T3 cells induces elevation of tyrosine phosphorylation

To determine whether Balb/c 3T3 respond to stimulation with both FCS and LPA, confluent cells were serum-starved and then stimulated with either 0.2% FCS or 100 ng/ml LPA. Equal amounts of lysate were resolved by SDS-PAGE and the phosphotyrosine profile analysed by Western blotting with an anti-phosphotyrosine antibody (Fig. 3.19). Although the serum-starved Balb/c 3T3 cells exhibit a higher level of background phosphotyrosine than serum-starved Swiss 3T3 cells, this may be a reflection of the shorter starvation period. Both FCS and LPA stimulate increased tyrosine phosphorylation of proteins of molecular weight 210, 116 and 60-70 kDa. Tyrosine phosphorylation of other proteins of 110 kDa and 80/85 kDa is also evident. Interestingly a phosphorylated band is evident at between 55-60 kDa which is also present in serum-starved Swiss 3T3 fibroblasts and may warrant further investigation.
Figure 3.17. Serum deprivation and stimulation of Balb/c 3T3 leads to an increase in tyrosine phosphorylation of cellular proteins. Confluent Balb/c 3T3 mouse fibroblasts were serum-starved for 30 minutes and 2 hours, then stimulated with 0.2% FCS for 20 minutes. Cells were lysed into protein loading buffer and equal amounts of lysate Western blotted with an anti-phosphotyrosine antibody (PT66). As always equal blotting and loading was checked by staining the nitrocellulose with Ponceau-S. Molecular weight markers (kDa) are shown on the left hand side of the blot.
1. Phosphotyrosine

2. Paxillin

3. Vinculin

Figure 3.18. Serum-stimulation of starved Balb/c 3T3 leads to the formation of actin stress fibres and focal adhesions. Balb/c 3T3 were serum-starved for 1 hour 45 minutes and then stimulated for 20 minutes with 0.2% FCS. Serum-starved cells (AB, EF, IJ) and stimulated cells (CD, GH, KL) were fixed and double stained for either F-actin (A,C,E,G,I,K) and phosphotyrosine (B,D), paxillin (F,H) and vinculin (J,L). Magnification bar represents 5 μm.
Figure 3.19. Both LPA and FCS stimulate tyrosine phosphorylation of specific cellular proteins in serum-starved Balb/c 3T3 cells. Balb/c 3T3 were serum-starved for 1 hour 45 minutes (0), and then stimulated with either 0.2% FCS (FCS 20) or 100 ng/ml LPA (LPA 20) for 20 minutes and lysed into protein loading buffer. Equal amounts of lysate were resolved by SDS-PAGE and Western blotted with an anti-phosphotyrosine antibody (PT66). Equal blotting and loading was checked by staining the nitrocellulose blot with Ponceau-S. Molecular size markers (kDa) are represented on the left hand side of the blot.
pp125Fak and paxillin show increased tyrosine phosphorylation upon stimulation of serum-starved Balb/c 3T3 cells with both FCS and LPA

Serum-starved Balb/c 3T3 cells were stimulated with both FCS and LPA and the lysates subjected to immunoprecipitation with an anti-phosphotyrosine antibody. The precipitate was then resolved by SDS-PAGE and the presence of pp125Fak and paxillin probed by Western blotting with antibodies against each protein (Fig. 3.20A,B and Fig. 3.21A,B). Markedly more pp125Fak and paxillin were precipitated from lysates of cells stimulated with either FCS or LPA.

To confirm that the two proteins were indeed tyrosine phosphorylated, each protein was directly immunoprecipitated from lysates of serum-starved cells, or cells stimulated with LPA. The precipitate was resolved by SDS-PAGE and the proteins detected by Western blotting with an anti-phosphotyrosine antibody (Fig. 3.22A and 3.23B). Again significantly more tyrosine phosphorylated protein was present in samples from stimulated cells. The blots were stripped and reprobed with antibodies against both pp125Fak and paxillin to determine the levels of each protein present on the blot. Although less pp125Fak is present in the LPA-stimulated track (Fig. 3.22B) this has been included to emphasise the level of stimulation of pp125Fak phosphorylation observed. Levels of paxillin were approximately equal in both tracks (Fig. 3.23B). This data confirms that both pp125Fak and paxillin tyrosine phosphorylation increases during the adhesive response, in this case upon stimulation of Balb/c 3T3 cells with LPA.

Genistein, a tyrosine kinase inhibitor, reduces LPA-mediated focal adhesion and stress fibre formation

The data presented raises the possibility that the formation of focal adhesions and stress fibres induced by LPA is dependent on the action of one or more tyrosine kinases. Burridge and co-workers (1992) have described a requirement for a tyrosine kinase sensitive to the inhibitor herbimycin A when cells spread on fibronectin. Herbimycin A is a tyrosine kinase inhibitor that preferentially targets src family tyrosine kinases (Uehara et al., 1989; Fukazawa et al., 1990). When cells are incubated with inhibitor overnight and then plated on fibronectin in the presence of the inhibitor, they remain rounded and do not exhibit tyrosine phosphorylation of pp125Fak or paxillin. To ascertain whether tyrosine kinases are essential for the LPA/rhoA-mediated formation of focal adhesions and stress fibres, we have also employed a number of tyrosine kinase inhibitors.

The effect of herbimycin A on the ability of FCS to stimulate the formation of stress fibres and focal adhesions was initially assessed. Although Swiss 3T3 cells were serum-starved in the presence of 875 nM herbimycin A, this appeared to have little effect on the ability of serum to stimulate the formation of either actin stress fibres or vinculin-containing focal
adhesions (data not shown). This is despite using the inhibitor as described by Burridge et al. (1992). This implies that the tyrosine kinase(s) essential for LPA- and integrin-mediated formation of focal adhesions and actin stress fibres may not be identical.

The effects of a second tyrosine kinase inhibitor genistein were therefore assessed (Akiyama et al., 1987). Serum-starved Swiss 3T3 cells were incubated with 2 μg/ml genistein for 30 minutes prior to stimulation with LPA (Fig. 3.24). Cells treated with genistein exhibited a marked decrease in the ability of LPA to induce the formation of actin stress fibres (Fig. 3.24E,G) or phosphotyrosine-containing focal adhesions (Fig. 3.24F,H) compared to control cells (Fig. 3.24C,D). However, the inhibition observed was not complete as some cells were still able to form a few actin filaments (Fig. 3.24G) and most possessed small phosphotyrosine clusters (Fig. 3.24H). These phosphotyrosine clusters were distributed over the ventral surface of the cell, but are distinct from the phosphotyrosine-containing adhesions seen in control cells. The presence of the phosphotyrosine clusters suggests that rather than blocking the response, genistein impairs the formation of mature focal adhesions. The data indicates that a genistein-sensitive tyrosine kinase involved in mediating the LPA response is required to elicit the formation of actin stress fibres and focal adhesions. This observation has been corroborated by Ridley and Hall (1994) who also demonstrated that genistein inhibits LPA-stimulation of serum-starved Swiss 3T3 cells. In that study the inhibitor was used at higher concentrations of 10 and 30 μg/ml, at which it was more effective at blocking any response. In summary there appears to be a requirement for a genistein-sensitive kinase in mediating signals downstream from rhoA, but not for the herbimycin A-sensitive kinase necessary for cells to spread on fibronectin.

3.2.15 pp125Fak exhibits kinase activity in serum-starved cells

The formation of focal adhesions stimulated by LPA and bombesin in serum-starved Swiss 3T3 cells and Balb/c 3T3 cells is associated with tyrosine phosphorylation and the activity of unidentified tyrosine kinases. If pp125Fak is indeed the tyrosine kinase responsible for generating the tyrosine phosphorylation associated with such stimulation, then a concomitant increase in its kinase activity should be observed upon stimulation of starved cells. To assess the kinase activity of pp125Fak in serum-starved and stimulated cells, an immune-complex kinase assay was employed. This assay is essentially dependent on the fact that pp125Fak autophosphorylates (Eide et al., 1995; Wilson et al., 1995). pp125Fak was immunoprecipitated from cell lysate and the ability of the pp125Fak (as an immune-complex immobilised on agarose beads) to autophosphorylate was assayed by incubation in a kinase buffer in the presence of magnesium and γ32P-ATP. Any kinase activity in the precipitate directed against pp125Fak will therefore incorporate 32P into the precipitated pp125Fak (described in detail in Materials and Methods) (Wilson et al., 1995). Pp125Fak was immunoprecipitated from serum-starved and LPA-stimulated Swiss 3T3 cells and the
Figure 3.20. Increased pp125Fak is immuneprecipitated by an anti-phosphotyrosine antibody from lysates of stimulated Balb/c 3T3. Balb/c 3T3 cells were serum-starved (0) and then stimulated with either 0.2% FCS (FCS, panel A) or 100 ng/ml LPA (LPA, panel B) for 20 minutes (20) and lysed into RIPA buffer. Tyrosine phosphorylated proteins were immuneprecipitated from the entire lysate and the levels of pp125Fak present in each phosphotyrosine precipitate determined by Western blotting with an anti-pp125Fak antibody. Molecular weight markers (kDa) are represented on the left hand side of each panel.

Figure 3.21. Increased paxillin is immuneprecipitated by an anti-phosphotyrosine antibody from lysates of stimulated Balb/c 3T3. Balb/c 3T3 cells were serum-starved (0) and then stimulated with either 0.2% FCS (FCS, panel A) or 100 ng/ml LPA (LPA, panel B) for 20 minutes (20) and lysed into RIPA buffer. Tyrosine phosphorylated proteins were immuneprecipitated from the entire lysate and the amounts of paxillin present in each phosphotyrosine precipitate determined by Western blotting with an anti-pp125Fak antibody. Paxillin is present at 66 kDa while the precipitating immunoglobulins are visible at 50 kDa. Molecular weight markers (kDa) are represented on the left hand side of each panel.
Figure 3.22. *pp125Fak becomes directly tyrosine phosphorylated when serum-starved Balb/c 3T3 are stimulated with LPA.* Balb/c 3T3 were serum-starved (0) and stimulated with 100 ng/ml LPA for 20 minutes (20) and the cells lysed into NP40 lysis buffer. *pp125Fak* was then directly immuneprecipitated from the entire lysate, the precipitate resolved by SDS-PAGE and then initially analysed by Western blotting with an anti-phosphotyrosine antibody PT66 (panel A). The blot was then stripped and reprobed with an anti-*pp125Fak* antibody (panel B) to determine the amounts of the protein present in each track. Molecular size markers (kDa) are represented on the left hand side of the blot.

Figure 3.23. *Paxillin becomes directly tyrosine phosphorylated when serum-starved Balb/c 3T3 are stimulated with LPA.* Balb/c 3T3 were serum-starved (0) and stimulated with 100 ng/ml LPA for 20 minutes (20) and the cells lysed into NP40 lysis buffer. Paxillin was then directly immuneprecipitated from the entire lysate, the precipitate resolved by SDS-PAGE and then initially analysed by Western blotting with an anti-phosphotyrosine antibody PT66 (panel A). The blot was then stripped and reprobed with an anti-paxillin antibody (panel B) to determine the amounts of the protein present in each track. Molecular size markers (kDa) are represented on the left hand side of the blot.
Figure 3.24. The tyrosine kinase inhibitor genistein inhibits stress fibre formation and reduces phosphotyrosine clustering. Serum-starved Swiss 3T3 cells were pretreated with 2 μg/ml genistein for 30 minutes prior to stimulation with 100 ng/ml LPA for 20 minutes. Cells were fixed and double stained for actin (A,C,E,G) or phosphotyrosine (B,D,F,H). Control cells serum-starved cells (A,B) were treated with DMSO, as were control LPA stimulated cells (C,D). Cells treated with both genistein and LPA (E,F and G,H) exhibit an impaired ability to from both actin stress fibres and phosphotyrosine containing focal adhesions.
Figure 3.25. pp125Fak isolated from serum-starved cells possesses tyrosine kinase activity. Panel A. pp125Fak was immuneprecipitated from the entire lysates of serum-starved (0) and LPA stimulated (100 ng/ml, 20 minutes) Swiss 3T3 fibroblasts (LPA 20). The kinase activity in both immuneprecipitates was assayed using a standard kinase assay (as described in materials and methods). The reaction was washed extensively and resolved by SDS-PAGE, the gel stained and dried down and the phosphorylated products detected by autoradiography.

Panel B. pp125Fak was immuneprecipitated from the entire lysates of serum-starved (0) and bombesin stimulated (50 ng/ml, 5 minutes) Balb/c 3T3 fibroblasts (Bomb. 20). The kinase activity in both immuneprecipitates was assayed using a standard kinase assay (as described in materials and methods). The reaction was washed extensively and resolved by SDS-PAGE, the gel stained and dried down and the phosphorylated products detected by autoradiography.

Panel C. Tyrosine phosphorylated proteins were immuneprecipitated from the entire lysates of serum-starved (0) and LPA stimulated (100 ng/ml 20 minutes) Swiss 3T3 fibroblasts (LPA 20). The kinase activity in both immuneprecipitates was assayed using a standard kinase assay (as described in materials and methods). The reaction was washed extensively and resolved by SDS-PAGE, the gel stained and dried down and the phosphorylated products detected by autoradiography.
kinase activity of the lysate assayed (Fig. 3.25A). pp125Fak precipitated from both serum-starved and LPA-stimulated cells became substantially labelled under the conditions of assay, though the pp125Fak isolated from LPA-stimulated cells appeared slightly more active. This result indicates that pp125Fak isolated from serum starved cells is active and can autophosphorylate. To confirm this observation, the pp125Fak kinase activity was assayed in serum-starved and bombesin-stimulated Balb/c 3T3 cells (Fig. 3.25B). Again substantial pp125Fak kinase activity could be detected in the serum-starved cells, which was slightly elevated upon stimulation of the cells with bombesin. These results were consistent between a number of independent experiments. This confirms that a pp125Fak kinase is active in serum-starved mouse fibroblasts.

It is possible that the pp125Fak kinase activity isolated from serum-starved cells is due to inadequate serum-starvation. This would result in a high background tyrosine phosphorylation of pp125Fak and possibly higher than normal kinase activity. To determine whether this kinase activity occurred in the absence of in situ tyrosine phosphorylation, lysates were prepared from serum-starved and LPA-stimulated Swiss 3T3 lysates in parallel with those in panel A. The tyrosine phosphorylated proteins were then immunoprecipitated from these lysates with anti-phosphotyrosine antibody conjugated to agarose beads. The pp125Fak kinase activity in these precipitates were also assayed (Fig. 3.25C). Here autophosphorylation could only be detected in the precipitate corresponding to cells stimulated with LPA. This confirms that the kinase activity present in the serum-starved cells, observed by direct precipitation of pp125Fak, is not due to the presence of tyrosine phosphorylated kinase.

Taken as a whole these results imply that an active pp125Fak kinase activity is present in serum-starved cells, and that this activity is independent of the tyrosine phosphorylation of pp125Fak associated with activation of the kinase activity.

3.2.16 Discussion - Part 1

Work in this section describes the characterisation of two cell lines in which events regulating the assembly of focal adhesions can be conveniently studied. Swiss 3T3 and Balb/c 3T3 mouse fibroblasts lose their actin stress fibres and focal adhesions upon serum deprivation. The subsequent LPA/FCS-mediated rho-dependent formation of focal adhesions recruited all the focal adhesion components analysed to the nascent structures. Moreover new focal adhesions form rapidly, detectable after only two minutes by immunofluorescence. The formation of focal adhesions is associated with the tyrosine phosphorylation of cellular proteins including pp125Fak and paxillin. This tyrosine phosphorylation also develops rapidly, and can be detected after as little as 30 seconds stimulation with LPA. From the effects of tyrosine kinase inhibitors, the system appears to be dependent upon a genistein-sensitive tyrosine kinase, as this agent reduces the ability
of the cells to respond to LPA. It is not however inhibited by herbimycin A, a tyrosine kinase inhibitor known to block spreading of fibroblasts on ECM components. Interestingly in serum-starved cells, although pp125Fak is not tyrosine phosphorylated, it does appear to possess kinase activity as determined by immunocomplex assay measuring autophosphorylation.

Formation of focal adhesions
The focal adhesions formed upon stimulation of serum-starved mouse 3T3 fibroblasts with LPA and FCS are mature structures, containing the components found in the focal adhesions of cells normally maintained in serum. Examination of the time course of assembly suggests that clusters of cytoskeletal proteins are formed before substantial stress fibre formation can be detected. It appears that phosphotyrosine clusters are initially detected both within the body of the cell, and at the periphery (after 2 minutes), presumably localised on the ventral surface, while the initial clusters of both vinculin and paxillin are only detected at the cell periphery at the same time point. This may be due to one of two reasons. The first is that the ventral focal complexes are detected because the anti-phosphotyrosine antibody is more sensitive than the antibodies to either vinculin or paxillin. The anti-phosphotyrosine antibody recognises epitopes on numerous proteins and so would be expected to stain focal complexes more efficiently than antibodies that see only one particular protein within these clusters. The second explanation, and perhaps the more attractive, is that these complexes represent the precursors or nucleation centres for the newly forming focal adhesions. A situation can be envisaged such that the proteins responsible for initiating the formation of the adhesion and the nucleation of the actin stress fibre, form complexes over the ventral surface of the membrane, and redistribute to the periphery of the cell as they mature into focal adhesions associated with a newly assembled actin stress fibres. The phosphotyrosine clusters become localised to the cell periphery when fine actin filaments can be seen to form. It is unfortunate that certain other cytoskeletal proteins have not yielded to analysis by this method. Of particular interest in this regard are pp125Fak, tensin and talin. As the monoclonal antibody against pp125Fak shows poor cross-reactivity with the mouse protein, attempts to document the time course of recruitment of this protein has not yielded any consistent results. The same is true of talin, the antibody to which is polyclonal and gave high background staining. As a result the presence of small focal clusters could not be easily discerned. The available anti-chick tensin antibody shows no cross reactivity with mouse protein.

Generation of reagents to both mouse talin and tensin may well prove fruitful in identifying the components of focal adhesion precursors. It is possible that these two proteins play important roles in generating new actin stress fibres. As discussed previously talin is reported to be an actin nucleating protein (Kaufmann et al., 1991; Kaufmann et al., 1992), while tensin caps the barbed ends of actin filaments such that actin monomers can be
introduced at the barbed end while being anchored by tensin (Lo et al., 1992; Lo et al., 1994b). Upon stimulation of serum-starved cells, new actin stress fibres could be formed by two different mechanisms. The first is through the nucleation of new actin filaments at the sites of integrin clusters. Talin would be a prime candidate for a protein capable of generating actin nucleation at sites of new actin stress fibres formation, as it can nucleate actin filaments in vitro (Kaufmann et al., 1991; Kaufmann et al., 1992). The second method is through addition of actin monomers to a nucleated actin "primer". It is possible that the serum-starved cells adhere through a number of immature unclustered points of adhesion containing the minimal number of cytoskeletal protein required to maintain adhesion. Under these conditions the immature adhesive structures may also contain an actin nucleation centre, perhaps a short actin filament held and capped by tensin. This would allow the cell to promote rapid actin filament growth in response to external stimuli by increasing the local concentration of free actin monomers at the ventral membrane.

The formation of the peripheral mature adhesions could, in turn, depend upon the clustering of newly formed actin stress fibres by actin crosslinking proteins. Recent work by Nobes and Hall (1995) has demonstrated that microinjection of rhoA stimulates actin filaments formation in the presence of staurosporine, a kinase inhibitor that blocks focal adhesion formation. Conversely focal adhesions can form in the presence of cytochalasin D, which inhibits the formation of new actin stress fibres (Nobes and Hall, 1995b). This implies that de novo actin polymerisation is not required for focal adhesion formation and that focal adhesions are not required for actin polymerisation. However in the absence of focal adhesions the organisation of the actin filaments appears affected. Nothing is known of the change in actin polymerisation upon stimulation of serum-starved cells. It will be important to determine whether the formation of actin stress fibres occurs as a result of de novo synthesis of F-actin or whether they arise due to a reorganisation of already polymerised actin within the cell. This would also provide an indication as to the role that actin polymerisation and stress fibres play in determining the organisation of focal adhesions.

**Tyrosine phosphorylation of specific cytoskeletal proteins**

The phenotypic changes induced by LPA/FCS correlate with changes in tyrosine phosphorylation of specific cellular proteins. Western blotting with the anti-phosphotyrosine antibody PT66 reveals that proteins of apparent molecular mass of 116 kDa 80 kDa and 60-70 kDa show changes in tyrosine phosphorylation. A second anti-phosphotyrosine antibody, PY20, revealed that a number of other proteins of molecular weights 110 kDa and 130 kDa are also tyrosine phosphorylated. Less intense bands of 210 kDa, 180 kDa and 90 kDa are recognised by one or both of the antibodies. Also of interest is a phosphorylated band that migrates at 50 kDa, present in serum-starved lysates and recognised by both antibodies. This difference in the repertoire of proteins recognised
by PT66 and PY20 demonstrates that although anti-phosphotyrosine antibodies are invaluable for studying signal transduction events, they may not recognise every tyrosine phosphorylated residue on target proteins. This is a distinct disadvantage, if like, PT66, the antibody fails to detect crucial proteins, such as the 110 kDa and 130 kDa proteins.

Despite this, it is plain that the profile of tyrosine phosphorylation observed upon LPA-mediated assembly of focal adhesions is very similar to that originally observed by Burridge and co-workers (1992) in fibroblasts spreading on ECM components. As determined by immuneprecipitation, both pp125Fak and paxillin become directly tyrosine phosphorylated upon LPA-mediated adhesion formation, again correlating with the observations of Burridge et al. (1992). The patterns of these changes in tyrosine phosphorylation are consistent with findings of other groups who have also studied LPA-mediated stimulation of Swiss 3T3 cells (Ridley and Hall, 1994; Seufferlein and Rozengurt, 1994). The same tyrosine phosphorylation events are observed upon stimulation with bombesin, associated with a stimulation of stress fibres and focal adhesions. This again correlates with the published work of others (Sinnett-Smith et al., 1993; Zachary et al., 1993; Ridley and Hall, 1994).

Of the other proteins undergoing tyrosine phosphorylation, comparison with observations made when cells spread on fibronectin suggests that the 210 kDa protein is likely to be tensin (Bockholt and Burridge, 1993; the 110 kDa protein p110 AFAP (Kanner et al., 1991), the 80 kDa protein cortactin (Vuori and Ruoslahti, 1995); and the 130 kDa protein pp130cas (Nojima et al., 1995; Petch et al., 1995; Vuori and Ruoslahti, 1995). pp130cas has been identified as one of the proteins tyrosine phosphorylated upon stimulation of quiescent Swiss 3T3 with LPA and bombesin (Seufferlein and Rozengurt, 1994). The same changes in tyrosine phosphorylation and cytoskeletal reorganisation induced by FCS and LPA have been observed in Balb/c 3T3 serum-starved cells. Other groups have also demonstrated the same tyrosine phosphorylation events in RAT1 rat fibroblasts (Hordijk et al., 1994; Malarkey et al., 1995). These observations confirm that the changes in tyrosine phosphorylation upon stimulation of cells with, for example LPA, is consistent between a number of cell types and not just a characteristic of one individual cell line. The tyrosine phosphorylation changes are associated with a reorganisation of the cytoskeleton.

Analysis of the early tyrosine phosphorylation events associated with the formation of focal adhesions and actin stress fibres shows that the signalling events stimulated by LPA are extremely rapid. Tyrosine phosphorylation of cellular proteins can be detected as quickly as 30 seconds after stimulation, and indeed both pp125Fak and paxillin are similarly tyrosine phosphorylated. This suggests that the signalling events leading to the rho-mediated response are direct. These observations at the protein level correlate well with those made by immunofluorescence analysis. The early events of LPA-stimulation
upon the tyrosine phosphorylation of pp125Fak and paxillin have been studied in greater detail by Seufferlein and Rozengurt (1994). Pp125Fak appears to undergo a rapid increase in tyrosine phosphorylation within 1 minute of stimulation and then falls away to 60% of that of the initial burst of phosphorylation. Paxillin phosphorylation increases rapidly but steadily becoming maximal about 5-10 minutes after stimulation. This initial burst of tyrosine phosphorylation of pp125Fak occurs at the same time point at which the small ventral phosphotyrosine-containing focal clusters are detected (this study). It is possible that clustering of integrins brings active pp125Fak into close proximity allowing autophosphorylation to occur.

In vitro assay of the pp125Fak kinase activity reveals that in the serum-starved cells the kinase is active, and activity is only stimulated a maximum of two fold upon addition of LPA and bombesin (as determined by phosphorimage analysis). This result has been confirmed using both Swiss 3T3 and Balb/c 3T3 cell lines stimulated with both LPA and bombesin. This kinase activity occurs in the absence of substantial tyrosine phosphorylation of pp125Fak as the activity is not present in anti-phosphotyrosine precipitates prepared from serum-starved cells. Although the assay provides a crude qualitative measure of the pp125Fak kinase activity present in individual lysates, two obvious flaws can be identified. The first is that the assay does not eliminate the possibility that other kinases may co-precipitate with pp125Fak, for example, src family members, and that their activity may also be directed against pp125Fak. The second is that it is not possible to determine the amount of precipitated pp125Fak present in each reaction.

One interpretation is that in the serum-starved cells pp125Fak is associated with the membrane perhaps through an association with β1-integrin or talin (Chen et al., 1995; Ilic et al., 1995; Schaller et al., 1995) in immature adhesive complexes where it displays activity. A transmembrane anchored chimaeric pp125Fak has been shown to be constitutively active even upon mutation of tyrosine 397 the residue upon which pp125Fak autophosphorylates (Chan et al., 1994). Autophosphorylation at this residue is indicative of pp125Fak activation. pp125Fak may only autophosphorylate when the kinases are brought into close contact upon the clustering of integrins (Kornberg et al., 1991; Kornberg et al., 1992; Lipfert et al., 1992). Alternatively the autophosphorylation/tyrosine phosphorylation of pp125Fak observed on stimulation of cells with LPA and bombesin may be due to a decrease in the degree to which the protein is dephosphorylated.

It is not known whether pp125Fak is the, or one of the, tyrosine kinases responsible for generating the formation of focal adhesions and actin stress fibres. Examination of cells obtained from pp125Fak knockout mouse embryos reveals that focal adhesions can still form, but that the absence of pp125Fak reduces the mobility of cells and the turnover of
focal adhesions (Ilic et al., 1995). Furthermore, pp125Fak is not required for the assembly of actin stress fibres and focal adhesions in mouse aortic smooth muscle cells (Wilson et al., 1995). Although neither study demonstrates a cellular function for pp125Fak they do question whether the protein is indeed the tyrosine kinase required for focal adhesion formation. It is likely that pp125Fak phosphorylation is a result of clustering rather than the mediator. In platelets, an analogous system, pp125Fak tyrosine phosphorylation is a later event that occurs after the initiation of the adhesive response and the activation of other tyrosine kinases (Huang et al., 1993). By virtue of the fact that pp125Fak tyrosine phosphorylation is so tightly associated with adhesive responses, it is reasonable to speculate that it is responsible for mediating a fundamental signal associated with cell adhesion. The other obvious candidate for the adhesion associated kinase is the src family of protein tyrosine kinases. Although inhibitor studies have indicated that src family members are not involved in the LPA-mediated formation of focal adhesions and actin stress fibres (this study; Ridley and Hall, 1994) they are implicated in mediating cell spreading (Burrige et al., 1992). Moreover transforming variants of the src family members (Kellie et al., 1986) and disruption of the negative regulator of src, csk (Imamoto and Soriano, 1993; Nada et al., 1993; Nada et al., 1994; Bergman et al., 1995) all have profound effects upon the organisation of the cytoskeleton. This alone suggests that the role of the src family in adhesion regulation is an important one, though interestingly ablation of individual members of the src family does not have serious deleterious consequences. It is likely that cell adhesion is regulated by a number of presently unidentified tyrosine kinases.

The tyrosine phosphorylation of a number of cytoskeletal proteins have been described, which are associated with the formation of adhesive structures. A number of these phosphorylation events have been shown in vitro to regulate SH2-dependent interactions, recruiting kinases such as src family members which in turn phosphorylate target proteins on alternative residues. For example autophosphorylation of pp125Fak at tyrosine 397 results in recruitment of src and fyn which phosphorylate pp125Fak at several residues within the C-terminus (Cobb et al., 1994; Schaller et al., 1994; Eide et al., 1995). Likewise pp125Fak-mediated phosphorylation of paxillin creates a binding site for the SH2 domain of crk (Schaller and Parsons, 1995). However little is known how these tyrosine phosphorylation events co-ordinate to regulate the formation of the focal adhesions. Importantly two proteins known to be tyrosine phosphorylated in cells transformed with RSV, β1-integrin and vinculin, do not become tyrosine phosphorylated upon stimulation of serum-starved Swiss 3T3 cells. Moreover β1-integrin and talin have been shown not to undergo tyrosine phosphorylation upon spreading of fibroblasts on fibronectin (Bockholt and Burridge, 1993). This is consistent with the idea that tyrosine phosphorylation of certain structural cytoskeletal proteins disrupts fibroblast adhesion. Moreover it implies...
that other mechanisms are responsible for the recruitment of certain cytoskeletal proteins to the focal adhesion.

**Signalling pathways regulating the rapid formation of focal adhesions and stress fibres**

The pathways positively regulating the formation of focal adhesions have been probed using a number of tyrosine kinase inhibitors. The tyrosine kinase inhibitor herbimycin A has been shown to inhibit cell spreading on fibronectin (Burridge et al., 1992) but in this study did not have an effect on the LPA- or FCS-mediated formation of focal adhesions. Cells were treated with herbimycin A at the same concentration and for the same length of time previously shown to inhibit spreading of cells on fibronectin (Burridge et al., 1992). These same observations have been made independently (Ridley and Hall, 1994). This work further showed that the PDGF-stimulated actin reorganisation was inhibited by herbimycin A. These two pieces of evidence suggest that at least one kinase in the pathway linking LPA and integrins to the formation of focal adhesions and actin stress fibres is unique to the integrin signalling pathway. Interestingly, herbimycin A has been shown to be a potent inhibitor of src family kinases in vivo and in vitro which suggests that src family members may not be involved in regulating adhesive signals mediated by the activation of G-protein linked receptors for LPA and bombesin (Ridley and Hall, 1994).

The tyrosine kinase inhibitor genistein affects the LPA-mediated formation of adhesions and stress fibres. At low concentrations (2-10 μg/ml) it reduces the ability of cells to respond to LPA. Genistein acts by competitively inhibiting ATP binding. As the ATP-binding site is a conserved feature of all protein kinases, it is unsurprising that at higher concentrations (above 50 μg/ml) genistein shows inhibitory effects against serine/threonine kinases (Akiyama et al., 1987), though at the concentrations used in this study genistein preferentially inhibits tyrosine kinases. Genistein has been further shown to completely inhibit the LPA response when used at the higher concentration of 30 μg/ml and acts in a manner that also inhibits ability of microinjected rhoA to mediate formation of focal adhesions and actin stress fibres (Ridley and Hall, 1994). Treatment of cells with genistein inhibits the LPA- and bombesin-mediated tyrosine phosphorylation of a number of cellular proteins (66 and 110-130 kDa) one of which is pp125Fak (Ridley and Hall, 1994). The results indicate that the formation of actin stress fibres and focal adhesions is mediated by a genistein-sensitive kinase that acts downstream of rhoA (see Fig 3.39). It is not however known whether pp125Fak is sensitive to genistein. The genistein-sensitive tyrosine kinase must be important to maintaining the integrity of the actin cytoskeleton as treatment of normal Balb/c 3T3 cells with 10 μg/ml genistein in the presence of serum, has been observed to disrupt actin stress fibres (data not shown).

The effects of a number of other tyrosine kinase inhibitors on the LPA-mediated formation of focal adhesions have been tested. The cell permeable erbstatin analogue has yielded
differential effects on the LPA-mediated formation of focal adhesion and stress fibres when used at the same concentrations. At 10 μg/ml Ridley and Hall (1994) observed no effect on LPA-, FCS- or bombesin-mediated stimulation of stress fibres and focal adhesions though it did inhibit PDGF mediated ruffling, a process mediated by PI3 kinase and rac (Ridley et al., 1992; Nobes et al., 1995), but not rho. Conversely, Chrzanowska-Wodnicka and Burridge (1994), suggested that the erstatin analogue affected the FCS-mediated formation of adhesions and stress fibres. This remains as yet unresolved.

A second family of tyrosine kinase inhibitors the tyrphostins have been shown to inhibit the LPA-mediated formation of stress fibres and adhesions. The tyrphostins 47 and 25 have previously been used to inhibit pp125Fak phosphorylation in spreading cells (Romer et al., 1994), and affect the bombesin-stimulated tyrosine phosphorylation of pp125Fak (Seckl and Rozengurt, 1993). Tyrphostins are regarded as specific for tyrosine kinases, mimicking the phosphorylated tyrosine residue in the peptide chain. They have not been seen to show activity against PKA or PKC as do other inhibitors at high concentrations (Levitski, 1990). These compounds inhibit the response of cells to LPA and more specifically reduce the tyrosine phosphorylation of pp125Fak (Chrzanowska-Wodnicka and Burridge, 1994; Nobes et al., 1995). The tyrosine kinase sensitive to tyrphostin 25 has been shown to lie upstream of rho, as microinjection of activated rhoA into tyrphostin treated cells results in the formation of actin stress fibres (Nobes et al., 1995). It is tempting to speculate that this kinase is the kinase postulated to link the LPA-receptor and rho proteins (Moolenaar, 1994). The conflicting nature of results obtained by different groups highlights the caution with which results obtained from the use of inhibitors must be regarded. Inhibitors have however proved invaluable in identifying at least two putative tyrosine kinases that regulated the formation of focal adhesions and actin stress fibres in response to LPA-stimulation of Swiss 3T3 cells.

A number of studies have implied that rho directly stimulates the tyrosine phosphorylation of cellular proteins including pp125Fak. Long term incubation of cells with C3 transferase inhibits the tyrosine phosphorylation of pp125Fak in response to LPA and bombesin (Kumagai et al., 1993; Rankin et al., 1994). This must be regarded with a degree a caution as long term exposure to C3 transferase can result in the ADP-ribosylation of other proteins particularly rac (Anne Ridley, personal communication). Furthermore treatment of permeablized Swiss 3T3 cells with the non-hydrolysable GTP analogue, GTPyS, results in the tyrosine phosphorylation of, among other proteins pp125Fak (Seckl et al., 1995). These observations provide circumstantial evidence that rho activates the tyrosine phosphorylation of proteins associated with the formation of focal adhesions. How rho might stimulate a downstream tyrosine kinase is unclear. One possibility is through a direct interaction with the kinase. For example the downstream effector of ras, raf is activated by ras via a direct interaction which results in recruitment of
raf to the membrane (Vojtek et al., 1993; Marais et al., 1995). The second possibility is that rho mediates different downstream effects by interacting with a variety of effector molecules which couple it to signalling enzymes.

As discussed earlier, further exploitation of the microinjection approach has shown that downstream of rho the signal from the LPA receptor bifurcates, (Nobes and Hall, 1995b). This suggests that the rho-mediated formation of focal adhesion is mediated by the action of a genistein-sensitive kinase and a staurosporine-sensitive kinase. The actin polymerisation is controlled by the genistein-sensitive kinase and other unknown proteins. Candidates include the lipid-modifying enzymes regulated by rho such as PIP 5-kinase. Rho has been implicated in activating PIP 5-kinase in cell free assays (Chong et al., 1994). The idea that rho may mediate effects on the cytoskeleton through promoting the generation of lipid messengers such as PIP2 and PIP3 species is an attractive one. Actin sequestering proteins such as profilin release actin monomers to preferentially bind PIP2 (Stossel, 1993; Sohn et al., 1995). The generation of such lipid second messengers at localised regions of the cell would result in a rise in the local concentration of free actin available for insertion into a growing actin filament. It is important to consider that the activity of a number of the "structural" cytoskeletal proteins also appears to be affected by the presence of such acidic phospholipids in vitro. For example both vinculin and α-actinin are reported to interact with PIP2 (Fukami et al., 1994), with both proteins activated in the presence of the phospholipid (Fukami et al., 1992; Weekes et al., 1996). Unfortunately, examination of such phospholipid species immediately following stimulation of starved Swiss 3T3 cells with LPA, bombesin and PDGF does not reveal gross changes in the different phospholipid species examined. Only the levels of P(3,4)P2 and P(3,4,5)P3 change in response to PDGF (Nobes et al., 1995). The change in cellular phospholipid content of cells spreading on fibronectin has not been examined. These results were obtained by analyzing the lipid contents of whole cells, and do not entirely rule out the possibility that such lipid species are generated at, or localised to, sub-cellular locations where they activate events at the required position.

The effect of other enzymes activated by LPA-stimulation
Treating cells with LPA and bombesin is known to stimulate a number of cellular signalling events, any one of which may effect cell adhesion (a diagram summarising the LPA pathway is presented in chapter 1, Fig. 1.14). Both stimulate PKC and induce calcium mobilization, which is presumed to be through the activation of PLC (van Corven et al., 1989). LPA also induces a decrease in cAMP concentrations and an increase in activated GTP-bound ras, ultimately activating the MAP kinase pathway (van Corven et al., 1989; van Corven et al., 1993). Other enzymes that could be activated as a result of intracellular events stimulated by LPA include the cAMP dependent kinase PKA which has been shown to stimulate the dissolution of actin stress fibres and focal adhesions (Lamb et al.,
The intracellular effects of PLC action were mimicked using a combination of the calcium ionophore A23187 and phorbol ester treatment but did not stimulate the formation of actin stress fibres (Ridley and Hall, 1994). This implied that PLC was unlikely to play a role in actin stress fibre formation. However, by directly addressing the role of PLC with an inhibitor, U-73122, a second study showed that inhibition of PLC reduced the response of Swiss 3T3 cells to LPA (Chrzanowska-Wodnicka and Burridge, 1994). PLC has previously been suggested to induce the tyrosine phosphorylation of several proteins including pp125Fak (Hordijk et al., 1994; Moolenaar, 1994). This important discrepancy is yet to be resolved.

Reduction in cAMP levels and activation of the ras/MAP kinase pathway inducing DNA synthesis is stimulated by a pertussis toxin sensitive G-protein, G_{i} or G_{o}. Treatment of cells with enough pertussis toxin to inhibit these two G-proteins had no effect on the ability of the cell to form actin stress fibres (Chrzanowska-Wodnicka and Burridge, 1994; Ridley and Hall, 1994). As the fall in cAMP does not affect actin stress fibre formation, this also implies that PKA inactivation has no effect. To determine whether PKA affects the integrity of the actin cytoskeleton, cells were stimulated in the presence of the PKA activator forskolin, and as predicted no reduction in stress fibre formation was observed (Ridley and Hall, 1994). Another downstream effect of LPA-stimulation is the activation of PKC. Stimulation of PKC with phorbol ester does not stimulate the formation of actin stress (Ridley and Hall, 1994), although it is required for spreading of a number of cell types on fibronectin (Woods and Couchman, 1992; Vuori and Ruoslahti, 1993). Treating spreading cells with TPA stimulates increased tyrosine phosphorylation of pp125Fak, which is inhibited by the PKC inhibitor calphostin C (Vuori and Ruoslahti, 1993). PKC is thought to regulate the affinity of integrins for the underlying matrix by a process known as inside-out signalling. The involvement of PKC in mouse fibroblast adhesion will be addressed in Chapter 5.

The inhibitor approach has therefore shown that rhoA appears to be stimulated, through a tyrosine kinase-dependent pathway, downstream of the LPA receptor, independent of the other cellular targets of LPA receptor activation. This pathway appears sufficient to stimulate the formation of actin stress fibres and focal adhesions as microinjection of rhoA generated focal adhesions and stress fibres as efficiently as LPA (Ridley and Hall, 1992; Ridley and Hall, 1994). The possible involvement of PLC in stimulating the formation of focal adhesions and actin stress fibres is important and must be resolved. Activation of PLC need not be a consequence of the action of LPA, but rather may be a downstream effect of rho activation. Indeed rho proteins have been implicated in activating a number of lipid modifying enzymes such as PLD and PIP 5-kinase (Chong et al., 1994; Malcolm et al., 1994) which may also be involved in adhesion regulation. The necessity for further analysis of the role of lipid modifying enzymes in cell adhesion is important,
especially in the light of recent studies highlighting the involvement of lipids in regulating both actin polymerisation and the activity of various cytoskeletal proteins. A possible link between rho and PLC has arisen with the cloning of a 122 kDa protein that possesses both rhoGAP activity and the ability to stimulate PLC-81 (Homma and Emori, 1995). A diagram showing a representation of the signalling events associated with focal adhesion formation is presented at the end of this chapter (Fig 3.39).
3.3 Results Part 2: The role of tyrosine phosphatases in the regulation of cell adhesion

3.3.1 The tyrosine phosphatase inhibitor vanadyl hydroperoxide stimulates assembly of actin stress fibres and focal adhesions

If the stimulation of focal adhesions and stress fibres by LPA is indeed dependent on the action of tyrosine kinases, it would be expected that inhibiting the negative regulator of these kinases (tyrosine phosphatases) might also trigger the assembly process. Sodium orthovanadate is a broad range tyrosine phosphatase inhibitor which can be converted to a cell permeable form, vanadyl hydroperoxide (orthovanadate), by mixing equimolar amounts of hydrogen peroxide and sodium orthovanadate (see Materials and Methods) (Heffetz et al., 1990; Heffetz et al., 1992; Ha et al., 1994).

Serum-starved Swiss 3T3 cells were treated with both 50 µM and 100 µM orthovanadate for 20 minutes to inhibit orthovanadate-sensitive phosphatases. Cells were double stained for actin, and either phosphotyrosine (Fig. 3.26) or vinculin (Fig. 3.27). As usual serum-starved cells contained few actin stress fibres (Fig. 3.26A,C and Fig. 3.27A,C) and no phosphotyrosine- (Fig. 3.26B,D) or vinculin-containing focal adhesions (Fig. 3.27B,D). Cells treated with hydrogen peroxide alone exhibited a small degree of membrane ruffling, but no significant actin filament formation at both 50 and 100 µM concentrations (Fig. 3.26E,G and Fig. 3.27E,G), or no evidence for formation of either phosphotyrosine (Fig. 3.26F,H) or vinculin-containing (Fig. 3.27F,H) focal clusters. Cells treated with both 50 and 100 µM orthovanadate formed numerous stress fibres and also exhibited increased membrane ruffling (Fig. 3.26I,K and Fig. 3.27I,K). These stress fibres terminated in vinculin-containing focal adhesions (Fig. 3.26I,L). However, when orthovanadate treated cells were stained with an anti-phosphotyrosine antibody it revealed that the entire cell stained heavily for tyrosine phosphorylation at both concentrations (Fig. 3.27I,L). This demonstrates the activity of the inhibitor against a wide range of tyrosine phosphatases. Due to the high level of tyrosine phosphorylation within the cell it was not possible to identify any phosphotyrosine-containing focal adhesions. These results were consistent in two independent experiments.

Prolonged treatment of cells with 50 µM orthovanadate for up to 1 hour induced very prominent actin stress fibres, with the whole cell staining extremely strongly for phosphotyrosine-containing proteins. A number of cells exhibited degrees of membrane ruffling while others possessed a large number of filopodia-like structures which also stained heavily for phosphotyrosine. Cells possessing these structure appeared to have undergone slight contraction of the cell body (data not shown).
Figure 3.26. **Orthovanadate induces the formation of actin stress fibres and the tyrosine phosphorylation of numerous cellular proteins.** Serum-starved Swiss 3T3 cells were treated with either 50 μM (AB, EF, IJ) or 100 μM (CD, GH, KL) concentrations of the broad range tyrosine phosphatase inhibitor orthovanadate for 20 minutes. Control serum-starved cells (A,B and C,D), control cells treated with 50 μM (E,F) or 100 μM (G,H) hydrogen peroxide, and cells treated with 50 μM (I,J) or 100 μM (K,L) orthovanadate were fixed and double stained for actin (A,C,E,G,I,K) and phosphotyrosine (B,D,F,H,J,L). Magnification bar represents 5 μm.
Phosphotyrosine

50 µM Orthovanadate

100 µM Orthovanadate

Orthovanadate  H₂O₂  Serum-starved
Figure 3.27. Orthovanadate induces the formation of actin stress fibres and recruitment of vinculin to focal adhesions. Serum-starved Swiss 3T3 cells were treated with either 50 μM (AB, EF, IJ) or 100 μM (CD, GH, KL) concentrations of the broad range tyrosine phosphatase inhibitor orthovanadate for 20 minutes. Control serum-starved cells (A,B and C,D), control cells treated with 50 μM (E,F) or 100 μM (G,H) hydrogen peroxide, and cells treated with 50 μM (I,J) or 100 μM (K,L) orthovanadate were fixed and double stained for actin (A,C,E,G,I,K) and vinculin (B,D,F,H,J,L). Magnification bar represents 5 μm.
Figure 3.28. Orthovanadate stimulates the tyrosine phosphorylation of numerous cellular proteins. Serum-starved Swiss 3T3 cells (0) were treated with either 50 or 100 μM orthovanadate (Van) or control hydrogen peroxide (H₂O₂) for 20 minutes. The cells were then washed in PBS, lysed into protein loading buffer and equal amounts of lysate analysed for tyrosine phosphorylated proteins by Western blotting with an anti-phosphotyrosine antibody (PT66). To check that the blot was evenly transfered the nitrocellulose filter was stained with Ponceau-S. Molecular weight markers (kDa) are represented on the left hand side of the blot.
To analyse the proteins tyrosine phosphorylated in response to orthovanadate, serum-starved cells were treated with 50 and 100 μM hydrogen peroxide and 50 and 100 μM orthovanadate for 20 minutes (Fig. 3.28). Cells were scraped into protein loading buffer, equal amounts of lysate resolved by SDS-PAGE and the distribution of tyrosine phosphorylated proteins analysed by Western blotting. From the blot it is evident that both concentrations of orthovanadate induced tyrosine phosphorylation of numerous proteins, to such an extent that identification of a specific pattern of tyrosine phosphorylated proteins was not possible. Hydrogen peroxide on its own did not appear to induce tyrosine phosphorylation of any proteins detectable by this method.

The broad range tyrosine phosphatase inhibitor orthovanadate therefore induces the formation of actin stress fibres and vinculin-containing focal adhesions. It also induces the tyrosine phosphorylation of numerous cellular proteins. The results provide further evidence of the importance of tyrosine kinases and tyrosine phosphatases in regulating cell adhesion.

3.3.2 The tyrosine phosphatase inhibitor phenyl arsine oxide (PAO) stimulates assembly of actin stress fibres and focal adhesions

Although the formation of actin stress fibres and focal adhesions stimulated by orthovanadate demonstrates a role for tyrosine phosphatase in the assembly process, the broad target range of the inhibitor limits its use as a tool in studying adhesion formation. A second tyrosine phosphatase inhibitor, phenyl arsine oxide (PAO), was therefore used to further assess the role of tyrosine phosphatases in adhesion regulation. PAO exhibits activity against mainly membrane associated phosphatases (Garcia-Morales et al., 1990), and has been previously shown to induce the tyrosine phosphorylation of pp125Fak among other proteins in CHO cells (Defilippi et al., 1995). As a collaborative project with Dr. Francesco Retta (University of Torino, Torino, Italy) PAO was used to further evaluate the importance of tyrosine phosphatases in the assembly of focal adhesions.

Serum-starved Swiss 3T3 cells were treated with PAO dissolved in DMSO, at a variety of concentrations ranging from 0.1 μM to 5 μM for 10 minutes, and then fixed and double stained for actin and phosphotyrosine. Control serum-starved cells treated with the highest appropriate amount of solvent (DMSO) possessed little or no actin stress fibres and no phosphotyrosine-containing focal adhesions (Fig. 3.29A,B). Treatment with PAO induced a dose-dependent response, with small clusters of phosphotyrosine becoming evident when cells were treated with 0.1 μM PAO (Fig. 3.29D) but no evidence of actin polymerisation (Fig. 3.29C). Upon treatment with 0.5 μM PAO for 10 minutes some peripheral focal phosphotyrosine staining became evident, but none resembling a normal focal adhesion (Fig. 3.29F). No significant actin stress fibre formation was induced (Fig 3.29E). Some actin stress fibres were evident when cells were treated with 1.0 μM PAO,
while the cells stained very strongly for phosphotyrosine-containing focal adhesions (Fig. 3.29G,H). When treated with 5.0 μM PAO cells contained numerous randomly distributed actin stress fibres (Fig. 3.29I) and prominent phosphotyrosine-containing focal adhesions (Fig. 3.29J). Interestingly when cells were treated with the higher concentrations of PAO there appeared to be a contraction of the cell body. The cells also appeared to possess filopodia-like structures which could be retraction fibres. With higher concentrations or prolonged treatment with PAO, the cells were seen to completely round up and detach from the matrix. These observations were consistent in at least two independent experiments. The results show that the tyrosine phosphatase inhibitor PAO acts in a dose dependent manner initially inducing the formation of strong phosphotyrosine-containing focal adhesions, and at higher concentrations the formation of poorly organised actin stress fibres.

To assess whether these phosphotyrosine-containing adhesions, induced by treatment with PAO, contain specific cytoskeletal proteins, serum-starved cells were stimulated with 1 μM PAO for 10 minutes and double stained for actin, and either vinculin or paxillin (Fig. 3.30). PAO induced the recruitment of both vinculin (Fig. 3.30C,D) and paxillin (Fig. 3.30G,H) to focal adhesions. These focal adhesions resembled normal focal adhesions found in LPA treated cells, though again the nature of actin stress fibres was distinct from those induced by LPA.

The time course of recruitment of tyrosine phosphorylated proteins to focal adhesions by the action of PAO was analysed (Fig. 3.31). Serum-starved cells were treated with 1 μM PAO for 2, 5 and 10 minutes, while control serum-starved cells were treated with the highest appropriate amount of DMSO solvent for 10 minutes. DMSO again had no effect on the serum-starved starved cells (Fig. 3.31A,B). PAO had little effect after 2 minutes with the cells containing few actin stress fibres or phosphotyrosine-containing focal adhesions (Fig. 3.31C,D). By 5 minutes, PAO induced the formation of phosphotyrosine-containing focal adhesions though little change in the number of actin stress fibres occurred (Fig. 3.31E,F). After 10 minutes the cells possessed focal adhesions staining heavily for phosphotyrosine, but only a few disorganised actin stress fibres (Fig. 3.31G,H). PAO appears to induce focal clustering of tyrosine phosphorylated proteins, with no clustering evident by 2 minutes, but detectable 5 minutes after treatment. This differs from the time course observed upon stimulation of serum-starved Swiss 3T3 cells with FCS or LPA. This may reflect the fact that PAO has to penetrate the membrane barrier to exert its action, while the active factor of FCS and LPA interact with a membrane receptor, possibly allowing them to activate the signalling process more rapidly.

PAO therefore acts in both a time- and dose-dependent manner to induce the formation of focal adhesions and to a lesser extent actin stress fibres. It induces the recruitment of
Figure 3.29. **Phenyl arsine oxide (PAO) stimulates the formation of phosphotyrosine-containing adhesions and actin stress fibres.** Serum-starved Swiss 3T3 cells (A,B) were treated with 0.1 μM (C,D), 0.5 μM (E,F), 1.0 μM (G,H) and 5.0 μM (I,J) PAO for 10 minutes. PAO was used at a stock concentration of 10 mM. The cells were then fixed, permeabilised and double stained for actin (A,C,E,G,I) and phosphotyrosine (B,D,F,H,J). Control serum-starved cells were treated with the appropriate amount of DMSO. Magnification bar represents 5 μm.
Figure 3.30. PAO stimulated the recruitment of vinculin and paxillin to focal adhesions. Serum-starved cells (AB, EF) were stimulated with 1 μM PAO for 10 minutes. The cells were then fixed, permeabilised and double stained for actin (A,C,E,G) and either vinculin (B,D) or paxillin (F,H). Control serum-starved cells were treated with the appropriate amount if DMSO. Magnification bar represents 5 μm.
vinculin, paxillin and tyrosine phosphorylated proteins to focal adhesions, though it is less efficient at inducing the formation of actin stress fibres.

3.3.3 PAO induces the tyrosine phosphorylation of proteins of 116-130 kDa and 66 kDa
To identify the cellular substrates of the protein tyrosine phosphatase(s) inhibited by PAO, serum-starved cells were treated with a range of concentrations of PAO for 10 minutes from 0.01 nM to 0.5 μM (Fig. 3.32) and 0.5 μM to 5.0 μM (Fig. 3.33). Control serum-starved cells were treated with the highest appropriate amount of DMSO for 10 minutes. PAO stimulated the tyrosine phosphorylation of a similar spectrum of proteins to that induced in response to LPA, most notably a diffuse group of bands ranging from 116-130 kDa, possibly a triplet, and to a lesser extent another diffuse band of 66 kDa. A small degree of stimulation was observed with as little as 0.01 μM PAO, becoming more evident with 0.05 μM, increasing in a dose-dependent manner with up to 0.5 μM (Fig. 3.32A). This dose-dependent response is still evident at higher concentrations of 1.0 and 5.0 μM (Fig. 3.33A). When samples were analysed by blotting from a small gel tyrosine phosphorylation of paxillin (66 kDa) can be seen with 0.5 μM PAO (Fig. 3.32). However little tyrosine phosphorylation of the same protein is evident when the samples are analysed using a large gel (Fig. 3.33). This is common when analysing proteins of molecular weights of 116 kDa and above on large gels, as lower molecular weight proteins (particularly paxillin) appear to be less efficiently captured on the blot.

Studying the time dependency of the tyrosine phosphorylation induced by PAO (Fig. 3.34) showed that, unlike LPA, little stimulation occurred within 2 minutes, but with significant stimulation after 5 minutes, correlating with the observations made by immunofluorescence analysis. Stimulation of serum-starved cells with LPA induces detectable increases in tyrosine phosphorylation after only 30 seconds. The PAO-dependent stimulation of protein tyrosine phosphorylation was consistent in at least four similar experiments.

3.3.4 PAO prevents cytochalasin D induced focal adhesion disassembly
Treatment of subconfluent, non-quiescent Swiss 3T3 with 1 μM cytochalasin D leads to the loss of the majority of actin stress fibres and phosphotyrosine-containing focal adhesions (Fig. 3.35E,F), with any remaining phosphotyrosine staining localised to diffuse patches rather than distinct focal adhesions seen in untreated cells. When 5 μM PAO was added to the culture media at the same time as cytochalasin D, cytochalasin D induced a loss of stress fibres, but prevented the loss of focal adhesions (Fig. 3.35G,H). The phosphotyrosine-containing adhesions seen were slightly smaller than those found in normal cultured cells and the number and size of the actin stress fibres much reduced. Indeed, although the integrity of the focal adhesion is well preserved (Fig. 3.35H) the actin
Figure 3.31. Time course of PAO induced focal adhesions and stress fibre formation. Serum-starved cells (A,B) were stimulated with 1 μM PAO for 2 minutes (C,D), 5 minutes (E,F) and 10 minutes (G,H). The cells were then fixed permeabilised and double stained for actin (A,C,E,G) and phosphotyrosine (B,D,F,H). Control serum-starved cells were treated with the appropriate amount of DMSO. Magnification bar represents 5 μm.
Figure 3.32. Dose-dependent tyrosine phosphorylation of cellular proteins by PAO. Serum-starved cells were treated with a range of concentrations of PAO, 0.01, 0.05, 0.1, 0.5 μM for 10 minutes. The control serum-starved cells (0) treated with the highest appropriate amount of DMSO for 10 minutes. The cells were washed once in PBS, lysed into protein loading buffer and equal amounts of the lysate analysed for tyrosine phosphorylated protein by Western blotting with an anti-phosphotyrosine antibody (PY20) (panel A). The blot was then stripped and reprobed for equal loading with an anti-vinculin antibody (panel B). Molecular weight markers (kDa) are represented on the left hand side of each panel.
Figure 3.33. Higher dose-dependent tyrosine phosphorylation of cellular proteins by PAO. Serum-starved cells were treated with a range of concentrations of PAO, 0.5, 1.0, 5.0 μM for 10 minutes. The control serum-starved cells (0) treated with the highest appropriate amount of DMSO for 10 minutes. The cells were washed once in PBS, lysed into protein loading buffer and equal amounts of the lysate analysed for tyrosine phosphorylated protein by Western blotting with an anti-phosphotyrosine antibody (PY20) (panel A). The blot was then stripped and reprobed for equal loading with an anti-vinculin antibody (panel B). Molecular weight markers (kDa) are represented on the left hand side of panel A.
Figure 3.34. Time course of tyrosine phosphorylation of cellular proteins by PAO. Serum-starved cells (0) were treated with 1 μM PAO for 2 minutes (2 min) and 5 minutes (5 min). The cells were washed once in PBS, lysed into protein loading buffer and equal amounts of lysate analysed for tyrosine phosphorylation of individual proteins by Western blotting with an anti-phosphotyrosine antibody (PY20) (panel A). The blot was then stripped and reprobed for equal loading with an anti-vinculin antibody. Control cells were treated with solvent DMSO for the full 5 minutes. Molecular weight markers (kDa) are represented on the left hand side of panel A.
Figure 3.35. PAO protects focal adhesions from cytochalasin D induced disassembly. Normal cultured fibroblasts maintained in serum (A,B) were treated with 1 μM cytochalasin D for 10 minute (E,F). Other cells were either treated with 1 μM cytochalasin D plus 5 μM PAO for 10 minutes (G,H). Control cells were treated with 5 μM PAO for 10 minutes (C,D,). Cells were fixed, permeablised and stained for either actin (A,C,E,G) or phosphotyrosine containing proteins (B,D,F,H). Magnification bar represents 5 μm.
stress fibres (Fig. 3.36) appear to be poorly protected by PAO. This protection of focal adhesion integrity appears to be due to inhibition of disassembly. Cells pretreated with cytochalasin D for 10 minutes before treatment with PAO do not exhibit any phosphotyrosine-containing focal adhesions (data not shown). These observations were consistent in two independent experiments.

3.3.5 PAO induces tyrosine phosphorylation in non-quiescent Swiss 3T3 cells
The above data suggests that inhibition of the PAO-sensitive phosphatase can stimulate the formation focal adhesions, and the tyrosine phosphorylation of specific cellular proteins in serum-starved cells. When serum is removed from normal cultured fibroblasts and replaced with serum-free media there is a rapid loss in both actin stress fibres and phosphotyrosine- and vinculin-containing focal adhesions. After 10-15 minutes the majority of cells possess only a fraction of the stress fibres and focal adhesion present in the normal cultured cells, and by 45 minutes a high proportion of the population begins to resemble serum-starved cells with few actin stress fibres and focal adhesions (data not shown). To assess the effect of PAO on non quiescent cells, 70-80% confluent Swiss 3T3 monolayers were treated with 1 and 5 μM PAO for 10 minutes, in serum-free media. While control cells in serum-free media possessed few actin stress fibres and focal adhesions (Fig. 3.36A,B), fewer than those found in normal cultured cells. Cells treated with PAO showed a dose dependent increase in the amount of actin stress fibres and focal adhesions (Fig. 3.36C-J). Indeed at 5 μM the stress fibres and focal adhesions are more abundant than those found in normal cultured cells. These observations were consistent in two independent experiments.

Analysis of the tyrosine phosphorylated proteins within such cells revealed that again PAO induced tyrosine phosphorylation of the same spectrum of proteins of 116-130 kDa, 100 kDa, 80 kDa and 66 kDa in a dose dependent manner. The results appear to support the possibility that PAO inhibits a phosphatase associated with disassembly of focal adhesions and actin stress fibres and dephosphorylation of the phosphotyrosine-containing proteins migrating between 116-130 kDa and at 66 kDa. Moreover, treatment of serum-starved Swiss with optimal levels of LPA plus 1 μM PAO enhances the formation of focal adhesions and stress fibres emphasising the important role that the PAO-sensitive tyrosine phosphatase plays in regulating cell adhesion.

3.3.6 PAO-sensitive phosphatase activity is enhanced in serum-starved cells
Evidence presented above implies that the serum-starved state is controlled, at least in part, by the action of a tyrosine phosphatase. As part of a collaboration with Dr. Francesco Retta (University of Torino, Torino, Italy) the tyrosine phosphatase activity in cultured cells and serum-starved cells was compared using tyrosine phosphorylated pp125Fak and paxillin as substrates. Essentially tyrosine phosphorylated pp125Fak and paxillin were
generated by treating cultured Swiss 3T3 with 5 μM PAO, followed by immune-precipitation of each protein. The immune-complexed proteins were then incubated with appropriate diluted cell lysate prepared from Swiss 3T3 cells at 37°C, and the tyrosine phosphatase activity in lysates prepared from normal cultured and serum-starved Swiss 3T3 cells compared. Substrate conjugated to agarose beads was incubated with the lysate for 5 minutes at 37°C, washed extensively, and then boiled in protein loading buffer (Fig. 3.38A,B). The tyrosine phosphorylated pp125Fak and paxillin in each sample analysed by Western blotting with an anti-phosphotyrosine antibody (Fig. 3.38A). The increase in phosphatase activity is visualised by the decrease in tyrosine phosphorylation of pp125Fak (116 kDa) and paxillin (diffuse, broad band 66-75 kDa). Significantly more dephosphorylation of tyrosine phosphorylated pp125Fak and paxillin occurred when the substrate was incubated with serum-starved lysate, compared with that treated with lysate prepared from cells maintained in culture. This indicates a higher phosphatase activity present in cells deprived of serum for 16 hours. Interestingly, even the cells maintained in culture exhibit a degree of phosphatase activity compared to the untreated control starting substrate. The blot was stripped and reprobed with antibodies specific for pp125Fak and paxillin (Fig. 3.38B). Approximately an equal amount of pp125Fak was present in the untreated and cultured lanes, with an increased amount present in the serum-starved lane confirming that the decrease in pp125Fak phosphorylation was not due to less substrate being present in this assay mix. The stripping procedure is a particularly harsh process and removes a lot of the target protein. In this case it has removed a lot of the paxillin present on the blot making detection difficult. These observations were consistent between at least three similar experiments.

The time course of pp125Fak dephosphorylation was assayed (Fig. 3.38C). Lysate from cultured and serum-starved Swiss 3T3 were extensively diluted and the phosphatase assayed over 5, 10 and 15 minutes. The lysate from cultured cells (ct) possessed a little phosphatase activity against pp125Fak with the amount of tyrosine phosphorylated pp125Fak decreasing markedly over time. The activity of the phosphatase against pp125Fak in lysates from serum-starved cells (st) was substantially increased. After 10 and 15 minutes the phosphatase had dephosphorylated nearly all the substrate in starved lysate. Incubation of the lysate from serum-starved cells with both pp125Fak substrate and PAO prevented dephosphorylation confirming that the phosphatase was PAO-sensitive. PAO inhibits the phosphatase by forming a covalent bond with thiol groups at the active site of the enzyme. The mode of action of PAO can be prevented by an oxidizing agent such as dithiothreitol (DTT). As a negative control to demonstrate that the action of PAO was specific and DTT inhibitable, lysate was incubated in the presence of PAO plus DTT. All the substrate was dephosphorylated after 15 minutes incubation with starved lysate in the presence of both PAO and DTT.
Figure 3.36. **PAO maintains focal adhesions and phosphotyrosine-containing focal adhesions.** Normal cultured Swiss 3T3 fibroblasts were treated with PAO in the absence of serum for 10 minutes. Cultured control cells (A,B) were treated with the highest appropriate amount of DMSO for 10 minutes. Other cells were treated with 0.1, 0.5, 1.0 and 5.0 μM PAO. Cells were fixed, permeabilised and double stained for actin (A,C,E,G,I) and phosphotyrosine (B,D,F,H,J). Magnification bar represents 5 μm.
Figure 3.37. PAO enhances the tyrosine phosphorylation of cellular proteins in cultured fibroblasts. Normal subconfluent cultured fibroblasts were treated with PAO for 10 minutes in the absence of serum. Control cells (0) were treated with the highest appropriate concentration of DMSO for 10 minutes. Other cells were treated with 1 and 5 μM PAO. The cells were washed once in PBS, lysed into protein loading buffer and equal amounts of lysate analysed for tyrosine phosphorylated proteins by Western blotting with an anti-phosphotyrosine antibody PY20 (panel A). The blot was stripped and reprobed for equal loading with an anti-vinculin antibody. Molecular weight markers (kDa) are represented on the left hand side of panel A.
Serum-starved cells contain enhanced PAO sensitive phosphatase activity (results provided by Francesco Retta). The phosphatase activity in lysates prepared from cultured and serum-starved cells was compared using a tyrosine phosphatase assay with tyrosine phosphorylated pp125Fak and paxillin as substrates (see materials and methods). Tyrosine phosphorylated pp125Fak and paxillin immunocomplexed to agarose beads were incubated with cell lysates for 5 minutes at 37°C. The pellet washed and boiled into protein loading buffer. Control substrate immunoprecipitate was incubated in kinase buffer alone to account for possible phosphatase activity in the immunoprecipitate. The results were then visualised by detecting the tyrosine phosphorylated proteins by Western blotting with anti-phosphotyrosine antibody, PY20 (panel A). To determine the amount of tyrosine phosphorylated pp125Fak and paxillin in each track, the blot was stripped and reprobed with antibodies specific for both pp125Fak and paxillin (panel B), although stripping appears to have removed most of the paxillin.

To demonstrate the activity of the phosphatase was time dependent, lysates of cultured (ct) and serum-starved (st) cells were diluted and incubated with tyrosine phosphorylated pp125Fak for 5, 10 and 15 minutes. To demonstrate that this phosphatase activity was PAO sensitive 5 μM PAO was included with cell lysate from serum-starved cells (st + PAO) and incubated with substrate for 15 minute. As a further control to demonstrate the specificity of PAO inhibition, both 5 μM PAO and 5 mM DTT (st + PAO + DTT) were included with the starved lysate. Molecular weight marker (kDa) are represented on the left hand side of each panel.
A Phosphotyrosine

B pp125FAK/Paxillin

C Time course phosphotyrosine

5 min 10 min 15 min
ct st st ct st st + st +
st + st +
PAO PAO PAO DTT/PAO

116 97 116 97

43 66 43 66
The results indicate that lysates prepared from serum-starved cells contain elevated tyrosine phosphatase activity against tyrosine phosphorylated pp125Fak and paxillin compared to that present on normal cultured cells. This phosphatase dephosphorylated pp125Fak in a time-dependent manner and was specifically inhibited by the addition of PAO to the assay mixture. Collectively, the results suggest that a PAO-sensitive phosphatase is critically involved in regulating cell adhesion in Swiss 3T3 cells.

3.3.7 Discussion - Part 2

Data presented in this section highlights the important role that tyrosine phosphatases play in regulating the integrity of the actin cytoskeleton and the formation of focal adhesions. In serum-starved cells the formation of focal adhesions and actin stress fibres can be stimulated by the broad range PTPase inhibitor sodium orthovanadate in its cell permeable form pervanadate (referred to as orthovanadate). Treatment with this agent also induces the tyrosine phosphorylation of numerous cellular proteins. A second tyrosine phosphatase inhibitor PAO induces the formation of focal adhesions, but is less effective at stimulating the formation of actin stress fibres. PAO acts more specifically than pervanadate inducing the tyrosine phosphorylation of proteins of molecular weights 110-130 kDa and 66 kDa, the same spectrum of proteins tyrosine phosphorylated upon stimulation with LPA. In vitro analysis of the phosphatase activity in serum-starved and normal cultured cell lysates reveals that serum deprivation results in an elevation of PTPase activity capable of dephosphorylating pp125Fak and paxillin. This tyrosine phosphatase is inhibited by PAO.

The action of tyrosine phosphatases

It is important to determine whether the tyrosine phosphorylation associated with the formation of focal adhesions is dependent upon the activation of tyrosine kinases or the inhibition of PTPases. By inhibiting tyrosine phosphatases, orthovanadate generates the formation of vinculin-containing adhesions, providing strong evidence that not only is the action of tyrosine kinases responsible for the formation of focal adhesions and actin stress fibres in serum-starved Swiss 3T3 cells, but that PTPases are integral to downregulation of the signalling events associated with assembly of these structures. Due to the broad specificity of orthovanadate, numerous proteins became tyrosine phosphorylated making it impossible to identify possible proteins of interest by Western blotting.

Use of orthovanadate on other cell systems also indicates that tyrosine phosphatases (and by inference the action of tyrosine kinases) have profound effects on the cytoskeleton. Treatment of rat neutrophils induces reorganisation of the F-actin network and the generation of pseudopodia and lamellapodia as well increased protein tyrosine phosphorylation (Bennett et al., 1993). Orthovanadate also increases focal adhesions and actin stress fibres as well as the tyrosine phosphorylation of cellular proteins in human...
endothelial cells (Defilippi et al., 1995). Interestingly, in this study low doses of orthovanadate caused an initial decrease in the tyrosine phosphorylation of pp125Fak which also correlated with a decrease in filamentous actin and focal adhesions. This suggests that in CHO cells at least two tyrosine phosphatases affect the tyrosine phosphorylation of pp125Fak. The first, inhibited at low concentrations of pervanadate acts to increase the tyrosine phosphorylation of pp125Fak. The second, inhibited at higher concentrations induces dephosphorylation of pp125Fak. In Swiss 3T3 cells, orthovanadate appears to act against a PTPase upstream of rhoA, as microinjection of C3 transferase inhibits the orthovanadate-induced formation of actin stress fibres and focal adhesions (Nobes et al., 1995). This not only provides further evidence that a tyrosine kinase is required for the activation of rhoA, but also that PTPases are crucial to the negative regulation of cell adhesion. Although the use of orthovanadate provides evidence that both tyrosine kinases and PTPases regulate focal adhesion formation its broad specificity limits its potential as a tool to study adhesion regulation.

The second PTPase inhibitor PAO proved more interesting in this respect. PAO is a trivalent arsenic derivative, thought to target membrane bound phosphatases. Indeed it inhibits a membrane bound PTPase involved in insulin signalling and the T cell receptor-mediated tyrosine phosphorylation pathway (Garcia-Morales et al., 1990; Liao et al., 1991). Although PAO clearly induced the formation of phosphotyrosine-containing focal adhesions it was less efficient than orthovanadate at promoting the formation of actin filaments. That the phosphotyrosine clusters also contained both vinculin and paxillin implies that the adhesions formed are mature. The effect of PAO on focal adhesion formation is dose-dependent, though at high concentrations it induces rounding of cells. At lower concentrations it induces contraction of the cell body, and as a result the PAO-stimulated cells appear smaller than the equivalent cells stimulated with either LPA or FCS. The effect of PAO is also time dependent, with focal clusters of phosphotyrosine appearing between 2 and 5 minutes after stimulation, rather than by 2 minutes as occurs upon stimulation with FCS or LPA. This further confirms that PTPases are responsible for negatively regulating cell adhesion, inhibition of which can promote the recruitment of proteins to the focal adhesion. Interestingly, PAO potentiated the LPA-stimulation of serum-starved cells increasing the number of actin filaments and focal adhesions formed (data not shown), perhaps indicating that the PTPases acts in a pathway parallel with the tyrosine kinase-mediated pathway. However the actin filaments formed upon stimulation of cells with PAO alone do not resemble normal actin filaments. This suggests that the PAO-sensitive phosphatase is distinct from the phosphatase inhibited by orthovanadate. Orthovanadate induced the formation of numerous actin stress fibres morphologically identical to those stimulated by LPA. This is presumably because a PTPase acting upstream of rho is inhibited (Nobes et al., 1995), which stimulates all events downstream of rhoA. It will therefore be important to determine whether PAO acts upstream or
downstream of rho to induce focal adhesion formation. PAO has previously been observed
to have no effect on serum-starved Swiss 3T3 cells (Chrzanowska-Wodnicka and Burridge,
1994). However in that study the PAO was applied at a concentration of 50 μM, which in
the present study would have induced cell rounding.

The repertoire of proteins which exhibit increased tyrosine phosphorylation in response to
PAO treatment is far more restricted than that of orthovanadate, and indeed resembles that
induced upon LPA or FCS treatment of serum-starved cells. PAO is most effective at
stimulating the tyrosine phosphorylation of a group of proteins which migrate at 110-130
kDa. However, it is less effective at stimulating the enhanced tyrosine phosphorylation of
the 66 kDa protein which is likely to be paxillin, though an increase does occur at higher
doses of PAO. This difference in potency may reflect one of two things; that the
phosphatase targeted by PAO is more active against paxillin (66 kDa) than the 110-130
kDa proteins and is not fully inhibited by PAO, or that two or more phosphatases
differing in sensitivity to PAO are involved in adhesion regulation.

Disruption of cell adhesion and the actin cytoskeleton has previously been shown to
activate PTPase activity (Maher, 1993). The observation that cytochalasin D-induced
disassembly of focal adhesions can be prevented by addition of PAO may indicate that the
disruption of focal adhesions induced by cytochalasin D result in part through increased
phosphatase activity. Interestingly PAO has also been shown to prevents the loss of both
actin stress fibres and focal adhesions in CHO cells treated with cytochalasin D (DeFilippi
et al., 1995). This cytochalasin D-induced disruption of the cytoskeleton also correlated
with a decrease in the tyrosine phosphorylation of pp125Fak (DeFilippi et al., 1995). In
Swiss 3T3 cells, cytochalasin D has previously been reported to inhibit both bombesin-
and LPA-mediated rho activation, and subsequent tyrosine phosphorylation of pp125Fak
(Sinnett-Smith et al., 1993; Seufferlein and Rozengurt, 1994; Nobes and Hall, 1995b). It is
possible to explain this observation in terms of the activation of PTPases. Cytochalasin
D-mediated disruption of the cytoskeleton elevates PTPase activity and so prevents any
cellular tyrosine phosphorylation as a result of bombesin or LPA-stimulation.

An in vitro phosphatase assay clearly demonstrated not only that serum-starvation results
in an elevation in the PTPase activity capable of dephosphorylating pp125Fak and
paxillin, but also that the phosphatase(s) responsible is inhibited by PAO. Interestingly
the phosphatase can be detected, with lower activity, in the lysate from normal cultured
cells. In the context of understanding the processes that regulate the formation and
perhaps turnover of focal adhesions these observations are highly significant. They
suggest that the disruption of focal adhesions and actin stress fibres is associated with the
increased activity of a PTPase coupled, although serum-starvation does not induce a large
change in pp125Fak activity. Significantly removal of serum from cultured cells resulted in
a noticeable loss of adhesions and stress fibres, which could be inhibited by the addition of 
PAO. Indeed higher concentration of PAO increased the size and numbers of focal 
adhesions and stress fibres, as well as the tyrosine phosphorylation of specific cellular 
proteins. The observation that the removal of serum leads to this rapid decrease in stress 
fibres and focal adhesions implies that adhesion may be regulated through a fine balance 
between kinase activity and phosphatase activity. Furthermore, it suggests that kinases 
and phosphatases involved in this regulation are extremely sensitive to changes in the 
extacellular environment. It would be informative to determine whether removal of serum 
from Swiss 3T3 cells, which induces a decrease in cytoskeletal structures, also rapidly 
reduces the tyrosine phosphorylation of cellular proteins and a concomitant increase a 
PAO-sensitive tyrosine phosphatase activity.

When serum-starved cells are stimulated with FCS, LPA or bombesin, kinase activity of 
pp125Fak increases slightly while the phosphatase activity is decreased. These changes 
together result in a greater overall change in phosphorylation state. A similar mechanism is 
employed to activate proteins involved in the kinase cascade that follows insulin 
stimulation (Liao et al., 1991; Mooney et al., 1992), and is an attractive way of rapidly 
activating kinase cascades. It is possible that a role of the PAO-sensitive PTPase is to act 
as a house-keeping phosphatase, being activated upon removal of a positive signal (ie 
removal of serum) ensuring rapid downregulation of cellular kinase activity. As the 
PTPase(s) becomes activated under conditions of cytoskeletal disruption, it may ensure 
that kinases such as pp125Fak are not active in the wrong cellular context. Rho-mediated 
formation of focal adhesions and actin stress fibres may therefore also require negative 
regulation of the PAO-sensitive phosphatase. Interestingly a serine/threonine phosphatase 
present in vascular smooth muscle cells, which dephosphorylates myosin light chain, is 
inhibited by GTPyS in a rho dependent manner (Noda et al., 1995). It is possible that the 
PAO-sensitive phosphatase may similarly be yet another downstream target of rho.
Alternatively FCS or LPA may decrease the activity of the PTPase independently of rho.
A third possibility is that the phosphatase is activated upon the removal of serum because 
it is inhibited by other factor present in serum other than LPA.

Little is known of the role that PTPases, and phosphatases in general, play in regulating 
the interaction of cells with the ECM. It is unclear which PTPases could be fulfilling the 
role of this adhesion related PTPase. A prime candidate must be the LAR family of 
transmembrane PTPases a member of which has been localised to focal adhesions (Serra-
Pages et al., 1995). A number of phosphatases which show homology to talin, namely 
PTPD1, PTPD2, PTPH1, PTPMSEG and PTPL1 are also interesting in this regard (Gu et al., 
1991; Yang and Tonks, 1991; Moller et al., 1994; Saras et al., 1994). Microinjection of 
the tyrosine phosphatase protein phosphatase type-1, but not type-2, leads to disruption 
of the integrity of the actin cytoskeleton in mammalian fibroblasts (Fernandez et al., 1990),
making this an alternative candidate. Understanding how the PTPase activity integrates with the positive action of tyrosine kinases to control focal adhesion assembly will be central to understanding the regulation of cell-ECM interactions. Despite this little effort has previously been directed towards determining the importance of PTPases in adhesion regulation.

Summary
Data presented in this chapter demonstrate that in Swiss 3T3 cells, LPA stimulation induces the rapid formation of focal adhesions and associated actin stress fibres, with a concomitant increase in the tyrosine phosphorylation of specific cellular proteins. Two of these proteins that show increase tyrosine phosphorylation are pp125Fak and paxillin. These same events occur upon stimulation of Balb/c 3T3 cells with FCS or LPA. The formation of focal adhesions is dependent upon a genistein-sensitive kinase, but is not affected by a second tyrosine kinase inhibitor herbimycin A. Importantly the formation of focal adhesions and stress fibres can be driven by inhibiting PTPase, indicating that tyrosine kinases drive the formation of adhesions and that PTPase negative regulate this process. This is borne out by the observation the removal of serum results in an increased PAO-sensitive phosphatase activity, and moreover that in serum-starved cells the pp125Fak kinase is active but not tyrosine phosphorylated. The known signalling events leading to the formation of focal adhesions and actin stress fibres are summarised in Fig. 3.39.
Figure 3.39. Schematic representation of pathways regulating cell matrix adhesion. The LPA-mediated and the fibronectin-mediated pathways are indicated. The positions of action of various inhibitors and activators described in the text are indicated in italics, agents which inhibit represented in red and agents which activate represented in green.
Chapter Four

Integrin-mediated assembly of focal adhesions and actin stress fibres
4.1 Introduction

In the previous chapter, evidence was presented that the LPA-mediated signalling pathway shares a number of common features with the fibronectin/integrin-mediated pathway. These two stimuli ultimately elicit similar effects on focal adhesion and actin stress fibre formation. Although serum-starved Swiss 3T3 cells lack focal adhesions and associated actin stress fibres, they remain attached to the substrate and appear to retain a well spread morphology (Barry and Critchley, 1994; Ridley and Hall, 1994). One outstanding question is whether the serum-starved cells adhere to the ECM via integrins in the absence of focal adhesions. The working hypothesis was that the cells in culture would adhere to either vitronectin or fibronectin, presumably in a manner dependent on the central cell-binding motif RGD. This peptide sequence is present in both fibronectin and vitronectin and is recognised by the integrin heterodimers α5β1 and α6β3 respectively. The possibility that serum-starved cells interact with the ECM in an RGD-dependent manner was probed using soluble peptide analogues of this cell-binding motif. If the cells adhere through engagement of this motif then it would be predicted that the cells would be detached from matrix by excess amounts of the soluble ligand.

Results

4.2 The GRGDS peptide induces detachment of serum-starved cells

The nature of the interaction between serum-starved cells and the substrate was probed using various synthetic peptides based on the RGD cell-binding motif of fibronectin. Initially the effects of the active peptide GRGDS, and a control peptide GRGES, on adhesion of serum-starved cells were tested. Serum-starved Swiss 3T3 were treated with various concentrations of peptide for the periods of time specified, then fixed and stained with crystal violet (Fig. 4.1). Both 1.0 mg/ml and 0.75 mg/ml GRGDS caused detachment of the cells from the matrix within 7-12 minutes of addition (Fig. 4.1D,F). GRGES had no effect on cell attachment at these concentrations even after 30 minutes of incubation (Fig. 4.1C,E). Similarly a second control peptide GRDGS did not round cells up when used at concentrations of 0.75 mg/ml for 30 minutes (data not shown). Incubation with 0.5 mg/ml GRGDS peptide for 30 minutes induced some rounding of cells in the monolayer, but no significant detachment (Fig. 4.1B), while the peptide had no effect on cell adhesion when used at 0.25 mg/ml (data not shown).

Serum-starved Balb/c 3T3 were also treated with the two peptides to confirm that this RGD-dependent adhesion was common to both cell-lines. The experiment was repeated essentially as previously described (Fig. 4.2), Balb/c 3T3 were serum-starved for 1 hour 45 minutes and treated with peptides at the same concentrations. The cells rapidly rounded up (7-8 minutes) in the presence of both 1.0 mg/ml and 0.75 mg/ml GRGDS (Fig. 4.2E,G) while GRGES had no effect at the same concentrations (Fig. 4.2F,H) even when incubated for up to 30 minutes (data not shown). Again treatment of cells with 0.5 mg/ml GRGDS
induced only a small degree of cell rounding (Fig. 4.2C), and had no effect on the cells when used at lower concentrations (data not shown).

The results clearly demonstrate that the serum-starved cells adhere to the ECM in an RGD-dependent manner. It is likely that the RGD motif of either vitronectin or fibronectin, is the ligand for integrins facilitating cell-matrix adhesion in serum-starved mouse fibroblasts in the absence of detectable focal adhesions or stress fibres.

4.3 Peptide analogues of the central cell-binding domain of fibronectin induce actin stress fibre formation in serum-starved Swiss 3T3 cells

When serum-starved cells are stimulated with LPA, a distinct morphological change can be seen when the cells are inspected using a phase contrast microscope (data not shown). Similar morphological changes were detected when the serum-starved cells were treated with the peptides GRGDS and GRGES, even at concentrations that did not induce cell rounding. The effect of the peptides within the same concentration range was examined by immunofluorescence. Treatment of cells with GRGDS (Fig. 4.3C,D) at 0.5 mg/ml stimulated the formation of prominent actin stress fibres and phosphotyrosine-containing adhesions. This stimulation also occurred when cells were treated with 0.25 mg/ml GRGDS. Interestingly treatment with the GRGES peptide at 0.5 mg/ml (Fig. 4.3E,F) also stimulated the formation of prominent actin stress fibres and phosphotyrosine-containing focal adhesions. This also occurred when the peptides were used at 0.25 mg/ml. The GRDGS peptide had no effect on the serum-starved cells when used at the same concentration under the same experimental conditions (Fig. 4.3G,H). GRDGS treated cells possessed no actin stress fibres and only a few small clusters of phosphotyrosine around the periphery of the cell. Due to the unexpected observation that the GRGES peptide stimulated focal adhesion formation a number of other peptide analogues were subsequently assayed for the ability to stimulate the formation of focal adhesions and stress fibres. Of the peptides assayed GRGD, SDGR and GGGD stimulated the formation of actin stress fibres and phosphotyrosine-containing focal adhesions at 0.5 mg/ml, although the degree of stimulation was variable. A fourth additional peptide DGR had no effect at 0.5 mg/ml. A full summary of the effects of different peptides is presented in Table 4.1. The data obtained suggests that soluble peptide analogues corresponding to the cell-binding motif of fibronectin are capable of inducing the formation of focal adhesions and actin stress fibres. However peptides that are variations of the defined binding motif for the fibronectin receptor are also capable of stimulating the cells.

Cells stimulated with 0.75 mg/ml GRGDS peptide were analysed, by immunofluorescence, as they rounded up (Fig. 4.4). Normally when stimulated with LPA cells display long actin stress fibres emanating from phosphotyrosine-containing focal adhesions, distributed around the periphery of the cell (Fig. 4.4A,B). Initially the peptide induced the formation
of actin stress fibres and phosphotyrosine-containing focal adhesions (as shown in Fig. 4.3). However, as the cells became detached, the cell bodies contracted leaving retraction fibres, some anchored by phosphotyrosine-containing adhesions. This cell retraction paralleled a reduction in the number of peripheral phosphotyrosine-containing focal adhesions (Fig. 4.4D,F) which were replaced in some cases with small phosphotyrosine clusters within the body of the cell, distributed over the ventral surface. Cell retraction also paralleled a disruption of the organised actin stress fibres (Fig. 4.4C,E). As the cell body contracted further disruption of the organised actin cytoskeleton occurred, with rounded cells staining brightly with phalloidin, and possessing no recognisable actin stress fibres or focal adhesions (Fig. 4.4C,D,E,F).

4.4 GRGDS-induced formation of actin stress fibres is rho-dependent
This experiment was performed in collaboration with Dr. Anne Ridley (Ludwig Institute for Cancer Research, London). To establish whether the GRGDS peptide-induced formation of actin stress fibres was rho-dependent, serum-starved cells were microinjected with the rho inhibitor C3 transferase, prior to addition of the peptides. The ability of the GRGDS peptide to induce stress fibres formation in serum-starved Swiss 3T3 cells (Fig. 4.5A,B) was clearly inhibited by microinjection of C3 transferase (Fig. 4.5D). The effect of the GRGES peptide was similarly inhibited by C3 transferase (data not shown). Again the GRDGS did not stimulate the cells (Fig. 4.5C). The peptide-induced formation of focal adhesions and stress fibres is therefore dependent on rho. The simplest interpretation is that the integrin-mediated pathway leading to the formation of actin stress fibres and focal adhesions is dependent on rho.

4.5 Serum-starved Swiss 3T3 form actin stress fibres when spread on fibronectin
To provide further evidence that integrin-mediated assembly of actin stress fibres could be stimulated in serum-starved Swiss 3T3 cells, serum-starved cells were spread on fibronectin. Serum-starved cells were trypsinised and plated on fibronectin and stained for F-actin after 15, 30, 45 and 60 minutes (Fig. 4.6). After 15 minutes on fibronectin, the cells were rounded with the periphery of the cell staining for cortical F-actin, and membrane ruffles (Fig. 4.6A). After 30 minutes the cells appeared flatter, though still slightly rounded, with the cell body containing numerous fine actin filaments (Fig. 4.6B). After 45 minutes on fibronectin the cells became well spread and containing clear actin stress fibres (Fig. 4.6C), a morphology more similar to that of cells maintained in culture, which became more pronounced after 60 minutes (Fig. 4.6D). The data demonstrates that spreading on fibronectin stimulates actin stress fibre formation in serum-starved Swiss 3T3 cells.

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Figure 4.1. The effect of GRGDS and GRGES peptides on adhesion of serum-starved Swiss 3T3 cells. Swiss 3T3 cells were serum-starved for 16 hours and then treated with either GRGDS (B-F) or GRGES (C,E) peptides as follows; (A) serum-starved cells. (B) 0.5 mg/ml GRGDS, 30 minutes; (C) 0.75 mg/ml GRGES, 30 minutes. (D) 0.75 mg/ml GRGDS, 10 minutes. (E) 1.0 mg/ml GRGES, 10 minutes. (F) 1.0 mg/ml GRGDS, 8 minutes. Cells were fixed and stained with crystal violet (representative of more than 4 experiments). Bar 50 µm.
Figure 4.2. The effect of GRGDS and GRGES peptides on adhesion of serum-starved Balb/c 3T3 cells. Balb/c 3T3 cells were serum-starved for 1 hour 45 minutes and the treated with either GRGDS (A,C,E,G) or GRGES (B,D,F,H) as follows; (A) and (B) serum-starved cells. (C) 0.5 mg/ml GRGDS 30 minutes. (D) 0.5 mg/ml GRGES 30 minutes. (E) 0.75 mg/ml GRGDS 10 minutes. (F) 0.75 mg/ml GRGES 10 minutes. (G) 1.0 mg/ml GRGDS 8 minutes. (H) 1.0 mg/ml GRGES 10 minutes. Cells were fixed and stained with crystal violet (representative of more than two experiments). Bar represents 50 μm.
Figure 4.3. The effect of GRGDS and GRGES peptides on the formation of actin stress fibres and phosphotyrosine-containing focal adhesions in serum-starved Swiss 3T3 cells. Serum-starved Swiss 3T3 cells were treated with the peptides shown at a concentration of 0.5 mg/ml for 10 minutes, and the cells were stained for actin (A,C,E,G) and co-stained for phosphotyrosine (B,D,F,H). (A,B) Control serum-starved cells. Cells treated with (C,D) GRGDS, (E,F) GRGES and (G,H) GRDGS (representative of more than two experiments). Bar represents 5 μm.
Figure 4.4. The GRGDS peptide disrupts actin stress fibres and peripheral phosphotyrosine-containing focal adhesions in rounding cells. Serum-starved Swiss 3T3 cells were stimulated with LPA (A,B), or with 0.75 mg/ml GRGDS peptide (C,D,E,F). The cells were then incubated with the GRGDS peptide for 15 minutes until a proportion of the population had rounded up, fixed and stained for F-actin (A,C,E) and phosphotyrosine (B,D,F). Bar represents 5 μm.
Figure 4.5. Microinjection of C3 transferase into serum-starved Swiss 3T3 cells inhibits the ability of the GRGDS peptide to induce actin stress fibres. (Courtesy Anne Ridley) Serum-starved Swiss 3T3 cells (A) before and (B) 10 minutes after addition of the GRGDS peptide (0.5 mg/ml). Cells were also microinjected with C3 transferase (2 mg/ml) before addition of (C) GRDGS or (D) GRGDS (0.5 mg/ml). Cells were again incubated with peptide for 10 minutes prior to fixation and staining for F-actin. Microinjected cells were identified by co-injection of FITC-labelled anti-rat IgG. Arrows in C and D indicate injected cells. Scale bar represents 20 μm.
Table 4.1. Summary of the adhesion motif peptides tested and their cellular effects. The three main peptides used are represented in bold. The four other peptides were assessed only for their ability to stimulate the formation of stress fibres and phosphotyrosine containing focal adhesions at 0.5 mg/ml, to determine specificity of the effect of the peptides. They were not tested at higher concentrations to determine whether they could round cells up. Differential effects of these four peptides on the ability to stimulate adhesions and stress fibres are represented.

<table>
<thead>
<tr>
<th>Adhesion Motif Peptide</th>
<th>Detachment of serum-starved cells</th>
<th>Stimulation of stress fibres and adhesions</th>
<th>Other comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>GRGDS</td>
<td>above 0.5 mg/ml</td>
<td>above 0.25 mg/ml</td>
<td>Stimulates tyrosine phosphorylation of pp125Fak and paxillin. Inhibited by C3 transferase. Adhesions disappear as cell round up.</td>
</tr>
<tr>
<td>GRGES</td>
<td>no</td>
<td>above 0.25 mg/ml</td>
<td>Stimulates tyrosine phosphorylation of pp125Fak and paxillin. Inhibited by C3 transferase. At lower concentrations the degree of stress fibres and adhesion formation appear less than that induced by GRGDS.</td>
</tr>
<tr>
<td>GRDGS</td>
<td>no</td>
<td>none until used at over 1.0 mg/ml</td>
<td>Does not stimulate the formation of stress fibres and adhesions until used at high concentrations.</td>
</tr>
<tr>
<td>GRGD</td>
<td>not tested</td>
<td>at 0.5 mg/ml</td>
<td>Tested only for ability to stimulate at 0.5 mg/ml</td>
</tr>
<tr>
<td>SDGR</td>
<td>not tested</td>
<td>at 0.5 mg/ml</td>
<td>Tested only for ability to stimulate at 0.5 mg/ml</td>
</tr>
<tr>
<td>DGR</td>
<td>not tested</td>
<td>none at 0.5 mg/ml</td>
<td>Tested only for ability to stimulate at 0.5 mg/ml</td>
</tr>
<tr>
<td>GRGGDS</td>
<td>not tested</td>
<td>at 0.5 mg/ml</td>
<td>Tested only for ability to stimulate at 0.5 mg/ml</td>
</tr>
</tbody>
</table>
Figure 4.6. Fibronectin-stimulated formation of actin stress fibre in serum-starved cells spread on fibronectin. Serum-starved Swiss 3T3 were trypsinised into suspension and the trypsin inhibited with approximately 0.5 mg/ml trypsin inhibitor (Sigma). Cells were then allowed to spread onto coverslips coated with 50 μg/ml fibronectin (Sigma) for (A) 15, (B) 30, (C) 45 and (D) 60 minutes (representative of more than two similar experiments). Bar represents 5 μm.
4.6 Tyrosine phosphorylation of cellular proteins in serum-starved Swiss 3T3 induced by fibronectin

To determine whether adhesion of serum-starved Swiss 3T3 cells to fibronectin induced the tyrosine phosphorylation of specific proteins, cells were again trypsinised and then spread on fibronectin coated dishes (50 μg/ml). Equal amounts of whole cell lysate, prepared in protein loading buffer, were analysed for tyrosine phosphorylated proteins by Western blotting with an anti-phosphotyrosine antibody (Fig. 4.7A). Tyrosine phosphorylation of a number of cellular proteins was observed as early as 15 minutes after plating on fibronectin, most prominent of which were a complex of bands migrating between 116 kDa and 130 kDa, and to a lesser extent a faint doublet at 66 kDa. This tyrosine phosphorylation appeared to be maintained up to 60 minutes after spreading on fibronectin. Due to the faintness of this blot the 0, 15 and 60 minute time points were rerun with more sample (Fig. 4.7B). This shows more clearly the stimulation of tyrosine phosphorylation of proteins of 116 kDa, 130 kDa, though the 66 kDa complex of proteins is still faint. Equal volumes of lysate were run on a separate gel and probed for vinculin (Fig. 4.7C) to determine that approximately equal amounts of protein were present in each sample. The tyrosine phosphorylation of the 66 kDa protein varied between experiments. As can be seen in Fig. 4.12 this protein is clearly tyrosine phosphorylated, migrating above the 66 kDa marker.

In summary, fibronectin appears to stimulate the same cellular processes in serum-starved Swiss 3T3 cells as serum, LPA or bombesin, specifically the formation of actin stress fibres and the tyrosine phosphorylation of specific proteins. The tyrosine phosphorylation is activated upon engagement of the ECM, becoming evident before the cell has developed a fully spread phenotype. The events in serum-starved cells correlate with those observed for cells spread in the presence serum, although the degree of stress fibre formation is lower than in the presence of serum (Burridge et al., 1992).

4.7 Rho is involved in the fibronectin-induced formation of actin stress fibres

This experiment was performed in collaboration with Dr. Anne Ridley. To determine whether rho proteins are required for serum-starved cells to spread on fibronectin C3 transferase was introduced into serum-starved Swiss 3T3 cells by scrape loading (Morris et al., 1989). Scrape loading is an efficient and reproducible method of introducing C3 transferase into a population of Swiss 3T3 cells (Flinn and Ridley, unpublished data). The ability of the cells to spread and to form actin stress fibres when plated onto fibronectin was then assessed. Control cells scrape loaded in buffer alone began to spread on fibronectin within 30 minutes with much of the F-actin localised under the plasma membrane and in lamellapodia (Fig. 4.8A). By 3 hours, the cells had adopted a typical fibroblast-type morphology and contained numerous actin stress fibres (Fig. 4.8C). Cells loaded with C3 transferase were able to adhere to fibronectin, but after 30 minutes few if
any cells were spread (Fig. 4.8B). After 3 hours the majority of cells were still rounded, and showed diffuse staining for F-actin with evidence of numerous filopodia-like structures (Fig. 4.8D). Some cells within the population adopted a well spread morphology, but lacked actin stress fibres, with punctate staining for F-actin reminiscent of that seen in serum-starved cells. While scrape loaded cells showed clustering was observed in cells scrape loaded with C3 transferase (data not shown).

To quantitate the inhibitory effect of C3 transferase on the fibronectin-induced formation of actin stress fibres, cells were scrape loaded with C3 transferase or buffer alone, and the cells fixed and stained for F-actin 60, 120 and 180 minutes after plating onto a fibronectin-coated substrate. Between 80-90% of cells scrape-loaded with buffer alone had formed actin stress fibres within 60-120 minutes of plating onto fibronectin, whereas <5% of cells scrape-loaded with C3 transferase were able to do so (Fig. 4.9). The results clearly indicate that rho is required for the fibronectin-induced formation of actin stress fibres in Swiss 3T3 cells.

4.8 C3 transferase inhibits fibronectin-induced protein tyrosine phosphorylation
This experiment was performed in collaboration with Drs. Helen Flinn and Anne Ridley (Ludwig Institute, London.). It has been well documented that adhesion of cells to fibronectin leads to increased tyrosine phosphorylation of a number of specific proteins including pp125Fak and paxillin (Burrige et al., 1992). To establish whether rho is involved in generating the tyrosine phosphorylation of these proteins serum-starved Swiss 3T3 cells scrape-loaded with C3 transferase and plated onto fibronectin were analysed for phosphotyrosine-containing proteins by Western blotting. Adhesion of control cells to fibronectin produced the expected increase in protein tyrosine phosphorylation (Fig. 4.10). In contrast, cells scrape-loaded with C3 transferase showed no such increase in tyrosine phosphorylation. The results indicate that rho is an essential component of the integrin-mediated signal transduction pathway, and is involved in both the formation of focal adhesions and actin stress fibres, and the activation of tyrosine kinases.

4.9 Genistein inhibits spreading of Swiss 3T3 cells on fibronectin
It has been clearly established that the tyrosine kinase inhibitor genistein inhibits LPA-mediated formation of focal adhesions and actin stress fibres, inhibiting a tyrosine kinase that lies downstream of rhoA (Chapter 3; Ridley and Hall, 1994). It was of interest to determine whether this genistein-sensitive kinase lies downstream of rho in the integrin-mediated pathway or whether rho acts to transduce a signal along distinct signalling pathways determined by the source of the signal. Serum-starved cells were pretreated with 30 μg/ml genistein for 30 minutes before being trypsinised and plated on fibronectin in the presence of 30 μg/ml genistein (Fig. 4.11). Cells were spread on glass coverslips, precoated with 50 μg/ml fibronectin, for 30 and 60 minutes and stained for actin. The
morphology of cells treated with solvent DMSO (Fig 4.11A,B) appeared unaffected when compared to untreated cells (not shown), cells still being slightly rounded after 30 minutes (Fig. 4.11A) and becoming more spread after 60 minutes (Fig. 4.11B). Cells treated with genistein exhibited much reduced spreading after 30 minutes (Fig. 4.11C) and still had the same morphology after 60 minutes (Fig. 4.11D). The genistein treated cells had an irregular shape with many lamellapodia, and some exhibited a degree of filopodia formation. Serum-starved cells could obviously adhere to fibronectin in the presence of genistein, but could not generate a fully spread phenotype. The effect was also observed when cells were treated 10 μg/ml genistein, though the degree to which spreading was retarded was reduced.

4.10 The effect of genistein on fibronectin-induced tyrosine phosphorylation

To determine whether genistein inhibited the tyrosine phosphorylation of cellular proteins, serum-starved Swiss 3T3 cells were treated with 10 and 30 μg/ml genistein, spread on fibronectin in the appropriate concentration of inhibitor and the tyrosine phosphorylation analysed by Western blotting (Fig 4.12A). Cells spread in the presence of DMSO, the solvent genistein is dissolved in, exhibited the same degree of protein tyrosine phosphorylation stimulated in untreated cells, specifically increased tyrosine phosphorylation of proteins migrating at 116-130 kDa and above the 66 kDa marker. Genistein did not visibly inhibit fibronectin-induced tyrosine phosphorylation when added at a concentration of 10 μg/ml. A possible decrease could be seen in the tyrosine phosphorylation of a protein migrating above the 66 kDa marker in cells incubated and spread in the presence of 30 μg/ml genistein, with a smaller decrease in the proteins migrating at 116-130 kDa. Although this inhibition of tyrosine phosphorylation has been observed in one other independent experiment with 30 mg/ml genistein the data obtained is far from definitive, and is not consistent between experiments, with no significant reduction visible in a third. This reflects the difficulty associated with using the pharmacological approach to dissect signalling pathways. Further experiments are required to establish whether rho does indeed signal to the same signalling pathway downstream of integrins and the LPA-receptor.

4.11 Discussion

Previous data has demonstrated that the LPA- and bombesin-induced formation of focal adhesions and actin stress fibres in serum-starved Swiss 3T3 fibroblasts is dependent upon the small GTP-binding protein rho (Ridley and Hall, 1992). The downstream effects of rho activation in this system appears to involve the tyrosine phosphorylation of a number of cellular proteins including pp125Fak, paxillin and pp130 (Barry and Critchley, 1994; Ridley and Hall, 1994; Seufferlein and Rozengurt, 1994). Activating integrins by spreading fibroblasts on fibronectin also induces a marked increase in tyrosine phosphorylation of focal adhesion proteins including pp125Fak, paxillin, tensin and pp130
Figure 4.7. Fibronectin-stimulated tyrosine phosphorylation of cellular proteins in serum-starved Swiss 3T3 cells. Serum-starved Swiss 3T3 were trypsinised into suspension and the trypsin inhibited with approximately 0.5 mg/ml trypsin inhibitor. Equal number of cells were then either kept in suspension in a polypropylene tube, or allowed to spread on tissue culture plastic coated with 50 µg/ml fibronectin. After the appropriate length of time the cells were lysed into protein sample loading buffer and equal volumes were separated by SDS-PAGE (8%) and then Western blotted with an anti-phosphotyrosine antibody. (A) anti-phosphotyrosine Western of cells spread for 0, 15, 30, 45 and 60 minutes. (B) anti-phosphotyrosine Western of the cells spread for 0, 15 and 60 minutes. (C) anti-vinculin Western of each sample to show equal loading (representative of three similar experiments). Molecular weight markers are represented on the left of (A) and (B).
Figure 4.8. C3 transferase inhibits spreading and stress fibre formation in cells plated on fibronectin. (Courtesy Anne Ridley) Serum-starved cells were scrape loaded with buffer alone (A,C) or C3 transferase (B,D). Cells were immediately plated onto fibronectin and allowed to attach and spread for either 30 minutes or 3 hours. Cells were then fixed and stained for F-actin (representative of two experiments). Scale bar represents 20 μm.
Figure 4.9. Quantitation of the effect of C3 transferase on the formation of actin stress fibres in cells plated onto fibronectin. (Courtesy Anne Ridley) Serum-starved cells were scrape loaded with C3 transferase (+C3) or buffer alone (-C3), and the cells plated onto fibronectin. Cells were incubated at 37°C for the times indicated, and then fixed and stained for F-actin. For each time point, 100 cells were scored from 10 randomly selected fields for the presence of actin stress fibres.
Figure 4.10. Inhibition of fibronectin-induced tyrosine phosphorylation by C3 transferase. (Courtesy Helen Flinn) Serum-starved Swiss 3T3 cells were scrape loaded with buffer alone (lanes 1 and 2) or with 230 μg/ml C3 transferase (lane 3). Cells were maintained in suspension (lane 1) or plated on fibronectin for 30 minutes (lanes 2 and 3). Equal volumes of the cell lysates were separated by SDS-PAGE, transferred to nitrocellulose and probed with the anti-phosphotyrosine antibody 4G10 (UBI). The molecular weight markers (kDa) are indicated on the left of the blot.
Figure 4.11. Gensitein inhibits spreading of serum-starved Swiss 3T3 cells on fibronectin. Serum-starved cells were treated with either solvent DMSO (A,B,) or 30 μg/ml gensitein for 30 minutes (C,D) and then trypsinised into suspension. Cells were then spread on coverslips coated with 50 μg/ml fibronectin for 30 minutes (A,C) and 60 minutes (B,D) in the presence of DMSO or genistein, then fixed and stained for F-actin (representative of more than two similar experiments). Bar represents 5 μm.
Figure 4.12. The effect of genistein on the fibronectin-stimulated tyrosine phosphorylation in serum-starved Swiss 3T3 cells. Serum-starved Swiss 3T3 cells were either untreated or incubated with solvent DMSO, 10 µg/ml or 30 µg/ml genistein for 30 minutes. The cells were then trypsinised into suspension. Cells were kept in suspension (control-0), or spread on fibronectin for 60 minutes (60) in the presence of the appropriate agent. Cells were then taken into protein sample loading buffer and equal amounts of lysate seperated by SDS-PAGE (8%) and Western blotted with an anti-phosphotyrosine antibody (A). The blot was stripped and reprobed for vinculin to determine the loading in each lane. Molecular weight markers are represented on the left hand side of (A).
(Burridge et al., 1992; Bockholt and Burridge, 1993; Petch et al., 1995), and a concomitant formation of actin stress fibres and focal adhesions. Moreover, engagement of the ECM by integrins also induces an activation of the MAP kinase signal transduction pathway (Chen et al., 1994). Here, using peptide analogues of the central cell-binding motif, serum-starved Swiss 3T3 cells and Balb/c 3T3 cells were shown to adhere in an integrin-dependent manner. Evidence is also presented that directly demonstrate that the integrin- and LPA-mediated signalling pathways require rho proteins. A number of peptide analogues stimulated the formation of prominent actin stress fibres and phosphotyrosine-containing focal adhesions integrins in a manner inhibited by microinjection of C3 transferase. Spreading serum-starved cells on fibronectin also induces the formation of actin stress fibres and the tyrosine phosphorylation of cellular proteins. This integrin-mediated tyrosine phosphorylation and actin stress fibre formation were likewise inhibited by C3 transferase.

Upon the removal of serum, both focal adhesions and actin stress fibres are lost in a number of cell lines including Swiss 3T3 cells and Balb/c 3T3 cells. However, cells remained spread and adhered to the underlying matrix in the absence of adhesive structures as detected by immunofluorescent analysis of serum-starved cells (Barry and Critchley, 1994; Ridley and Hall, 1994). Unfortunately the lack of good mouse reactive reagents against β1-integrins has meant that the distribution of integrins in serum-starved and serum-stimulated cells could not be analysed. This is despite analysis with a number of polyclonal antibodies to both the cytoplasmic domain and the extracellular domain of β1-integrin (data not shown). To generate a mouse specific anti-β1 integrin antibody, a fusion protein corresponding to the cytoplasmic domain of mouse β1-integrin was constructed. However this resulted in expression of insoluble protein, making production of an antibody impossible. The failure to detect integrins by fluorescence in Swiss 3T3 cells may be because the epitopes recognised by the antibodies are masked in adherent cells. To investigate the role of integrins in maintaining adhesion of the serum-starved cells, the effect of peptides corresponding to the cell-binding motif in fibronectin were used to determine whether they adhered in an integrin-dependent manner. The ability of the GRGDS peptide, but not GRGES to detach serum-starved cells from the matrix provides strong evidence that serum-starved mouse fibroblasts adhere in an integrin-dependent manner, although no visible focal adhesions are present.

The serum-starved Swiss 3T3 cells have been studied by interference reflection microscopy (IRM) (data not shown). This technique enables regions of close contact between cells and the underlying substrate to be visualised as a dark region within the cell (Abercrombie and Dunn, 1975). Normally the focal adhesions in fibroblasts containing actin stress fibres can be visualised as dark distinct ovoid patches distributed around the periphery of the cell, similar in appearance to the focal adhesions visualised by
immunofluorescence. In serum-starved cells the ventral surface of the membrane appeared to be in close contact to the matrix, visualised as large dark regions on the ventral surface of the cell. This may suggest that the serum-starved cells adhere to the underlying matrix via dispersed patches of integrins rather than the tightly co-ordinated cluster of integrins, around which the structure visualised as the focal adhesion assembles.

Interestingly when the GRGDS peptide was used at concentrations below that required to round cells up, it was capable of stimulating the formation of actin stress fibres and focal adhesions. It seems that although the cells are adhering via integrins these adhesive links are not sufficient to activate the system. This stimulus, in Swiss 3T3 cells, can be provided by the subsequent addition of LPA or FCS. Binding of the GRGDS peptides to unoccupied integrins must also be sufficient to generate this signal. Peptides corresponding to the RGD cell-binding motif have previously been shown to activate the signal transduction pathways leading to platelet aggregation (Du et al., 1991) and to increase collagenase and stromelysin gene expression in fibroblasts (Werb et al., 1989). GRGDS peptide binding to the unoccupied fibronectin receptor, has been shown to sufficient to induce recruitment of this now occupied receptor to focal adhesions (LaFlamme et al., 1992). As soluble ligand binding is reported to activate the fibronectin receptor it can be hypothesised that driving activated integrins to sites of cell matrix contact in the serum-starved cell may well be enough to activate clustering into focal adhesions, and hence stimulate the system.

At higher concentrations the peptide presumably also begins to act as a competitive inhibitor of fibronectin-bound integrins, resulting in detachment of cells from the ECM. The rapid cell rounding effect may be a result of cooperation between the contractile response stimulated by the peptides and the weakening of the adhesive links, by competition for bound integrins. As a result the tension exerted by the newly formed actin stress fibres pulls the weakened cell-matrix contacts from the matrix resulting in cell retraction. Interestingly the GRGDS peptide has previously been shown to induce the dissociation of α-actinin and vinculin from the sites of focal contacts when used at high concentrations (Stickel and Wang, 1988). As the GRGDS peptide rounds up the serum-starved cell, the newly formed actin stress fibres lose their integrity, with a change in the number and distribution of the focal adhesions. In some cells small point clusters could be seen to form on the ventral surface within the body of the cell. These structures may represent adhesive links formed by the cell in response to disruption of the peripheral points of adhesion.

Surprisingly the GRGES peptide was also seen to stimulate the formation of focal adhesions and associated actin stress fibres in serum-starved cells, as were a number of other peptides that were subsequently tested. The only peptides found to be negative under
the conditions of assay were GRDGS and DGR. There is no obvious explanation for this effect. Importantly similar effects were seen with two batches of peptides synthesised independently and prepared independently. One possibility is that a number of these peptides are similar enough to the RGD motif to allow sufficient interaction with the receptor. Concentrating in the effect of the GRGES peptide, several explanations have been considered. The original literature suggests that this peptide is unable to bind to the fibronectin receptor (Pierschbacher and Ruoslahti, 1984) and does not support cell adhesion (Oba et al., 1988). However it has been shown to compete with fibronectin for binding to the fibronectin receptor isolated from placenta (Hautanen et al., 1989). The GRGESP peptide was 2000 fold less active than the GRGDSP, inhibiting fibronectin binding by 50% at concentrations of $10^{-4}$ M (Akiyama et al., 1985). In the present study the GRGES peptide was used at approximately $10^{-3}$ M, at which concentration it would be expected to interact with the fibronectin receptor in Swiss 3T3 cells. Whilst this concentration of GRGES may be able to activate the signalling process by occupying the unbound fibronectin receptor, it is presumably unable to compete cells off the matrix. The same may be true of the other control peptides, GRGGDS, GRGD and SDGR shown to stimulate serum-starved cells. These may also be similar enough to the RGD motif to interact with, but not act as a competitive ligand for integrins.

Alternatively the GRGES peptide may be interacting with another integrin heterodimer. The integrin $\alpha_4\beta_1$ has been reported to bind to both GRGDS and GRGES with equal affinity (Mould et al., 1991). This integrin is expressed predominantly in melanoma cells, lymphocytes and neural crest cells. It is also expressed in human dermal fibroblasts (Gailit et al., 1993) and rabbit synoval fibroblasts (Huhtala et al., 1995). The expression of $\alpha_4\beta_1$ integrin in Swiss 3T3 cells was investigated by both FACS (Garratt and Humphries, unpublished data) and Western analysis (this study, data not shown) with a rat anti-mouse $\alpha_4$ antibody. Both approaches revealed that there is no detectable $\alpha_4$ expressed in Swiss 3T3 cells. Given that $\alpha_4$ integrin can bind a variety of peptide ligands (Mould et al., 1991) it is not inconceivable that other uncharacterised integrins are capable of interacting with the peptide ligands used to challenge the serum-starved Swiss 3T3 cells. The fact that the peptide effect is inhibited by C3 transferase, and that a number of the peptides show differing effects on the cells, implies that the peptides act specifically to activate a common signalling pathway. This is supported by the observation that GRGDS and GRGES induced the tyrosine phosphorylation of the same profile of proteins as that induced by LPA treatment, namely a group of proteins migrating at 116 kDa and 66 kDa (data not shown). The simplest interpretation of the data is that integrin- and LPA-mediated formation of focal adhesions both require rho.

Integrins exist in a variety of activation states which depend upon both ligand occupancy and signals from the cytoplasmic face of the membrane. Both subunits of the heterodimer
contribute to ligand-binding and activation of integrins. The cytoplasmic tail of β1-integrins determine localisation to focal adhesions (Hayashi et al., 1990; LaFlamme et al., 1992). The α subunit cytoplasmic domain plays a regulatory role (O'Toole et al., 1994), mediated by conserved domains in the membrane proximal C-terminus (Ylanne et al., 1993). Similarly a conserved sequence, NPXY (single amino acid code) within the membrane proximal C-terminus of the β1-integrin has also been implicated in activation of the heterodimer (O'Toole et al., 1995). Although GRGDS peptide can drive unoccupied receptor into focal adhesions, presumably as a result of ligand binding (LaFlamme et al., 1992), whether the activated receptor can stimulate the clustering of dispersed fibronectin-bound receptor into focal adhesions, and the associated protein tyrosine phosphorylation is unclear.

The effect of soluble or immobilised ligands, and activating or non-inhibitory integrin antibodies on the activation state of integrins has been investigated using polystyrene beads coated with various ligands. These beads induce clustering of integrins on the dorsal surface of the cell which recruit cytoskeletal proteins to form strong transmembrane links (Miyamoto et al., 1995a; Miyamoto et al., 1995b). These complexes are analogous to focal adhesions, but as they are larger the presence of low abundance proteins can be detected by immunofluorescence due to the elevated local concentration of protein (Mueller et al., 1989; Plopper and Ingber, 1993; Lewis and Schwartz, 1995). Using coated beads to cluster integrins on cells spread on polylysine, Miyamoto and co-workers (1995a; 1995b) have identified a number of different transmembrane responses induced by integrins. These signals mediate the recruitment of numerous proteins to focal adhesion-like complexes formed on the dorsal surface of the cell. The components of these complexes have been analysed by immunofluorescence. Clustering integrins with beads coated with anti-integrin antibody induced the recruitment of a large number of signalling molecules including tensin, pp125Fak, cortactin, certain src family members, PLCγ, PI3 kinase, rhoA, rac1 and members of both the ERK and SAPK/JNK and MAP kinase pathways. Integrin occupancy with soluble peptide and antibody-induced clustering resulted in the additional recruitment of vinculin, talin and α-actinin along with the signalling elements described above. Interestingly recruitment of all the signalling proteins, except pp125Fak, were inhibited by the addition of tyrosine kinase inhibitors. Integrin clustering with a variety extracellular antibodies in the presence of the tyrosine kinase inhibitors genistein and herbimycin A resulted in the recruitment of tensin and pp125Fak to the bead cell interface. Alternatively, in the presence of tyrosine kinase inhibitors clustering and ligand occupation only induced the recruitment of tensin, pp125Fak, α-actinin, vinculin and talin to adhesive complexes. These observations imply that the activation state of the integrin regulates the recruitment of distinct groups of proteins to adhesion complexes, and furthermore that the action of tyrosine kinases sensitive to genistein and herbimycin A is required for the recruitment of a specific
subclass of proteins to adhesive complexes. Therefore receptor clustering alone is enough to induce tyrosine phosphorylation and the recruitment of pp125Fak and tensin to sites of adhesions, but receptor occupancy and clustering is required for the formation of a focal adhesion.

Interestingly Miyamoto and coworkers (1995b) further showed that integrin clustering stimulated the activation of the ERK and SAPK/JNK pathways. However all signalling events except that of the activation of SAPK/JNK were inhibited by cytochalasin D and tyrosine kinase inhibitors. The rho family members rho, rac and cdc42 have been shown to regulate the SAPK/JNK pathway (Hill and Treisman, 1995; Hill et al., 1995; Minden et al., 1995; Coso et al., 1995) and also are involved in progression through the cell cycle (Olson et al., 1995). From such studies (Miyamoto et al., 1995a; Miyamoto et al., 1995b), it is becoming obvious that integrins mediate a hierarchy of molecular responses, which not only affect the organisation of the adhesive machinery, but also the activation of intracellular signalling pathways.

This data does not however explain why the adhesion motif peptides are capable of stimulating the serum-starved Swiss 3T3 cells. In this system the absence of detectable focal adhesions implies that the integrins are not tightly aggregated. This is supported by the observation that the level of tyrosine phosphorylation is low. The observation that the GRGDS peptide could generate the formation of focal adhesions containing high levels of tyrosine phosphorylation suggests that ligand occupancy of unoccupied receptors is enough to mediate both integrin activation and cellular tyrosine phosphorylation.

Due to the confusing data obtained using the peptides, the relationship between rho and integrin-mediated signalling was investigated using an alternative approach. It has been previously shown that cells spread on fibronectin, in the presence of serum, exhibit stress fibre formation and the tyrosine phosphorylation of pp125Fak, paxillin, tensin and pp130 (Burridge et al., 1992; Bockholt and Burridge, 1993; Vuori and Ruoslahti, 1995). That serum-starved cells spread on fibronectin, form stress fibres and exhibit tyrosine phosphorylation of cellular proteins suggests that activation of integrins alone can stimulate the system. The activation is not however as pronounced as that observed in cells spread in the presence of serum (Burridge et al., 1992), suggesting that for full/optimal activation of stress fibres and focal adhesions, a signal is required from both integrins and serum components. Both the fibronectin-induced stress fibres and protein tyrosine phosphorylation are inhibited by scrape loading cells with C3 transferase confirming that rho is required for integrin-mediated signalling.

How rho and integrins are coupled is not known but the ability of genistein to affect cell spreading on fibronectin implies that the downstream mediators of rho may be common to
both the LPA- and integrin-mediated pathways. However analysis of the tyrosine phosphorylation of cellular proteins stimulated by integrin activation in the presence of genistein revealed that the tyrosine phosphorylation of cellular proteins was not substantially affected. This may imply that the genistein-sensitive kinase is required for cell spreading but that engagement of integrins is enough to stimulate the tyrosine phosphorylation of certain cellular proteins. Interestingly genistein was seen to inhibit the recruitment of a number of signalling proteins to focal complexes induced by integrin activation with coated polystyrene beads but not pp125Fak (Miyamoto et al., 1995a). An alternative explanation is that upon activation of integrins by fibronectin in the presence of genistein, pp125Fak is activated and so autophosphorylates, but that genistein targets an alternative tyrosine kinase required to generate a spread phenotype. This would explain how a distinct phenotypic change could be seen at the cellular level within the majority of the population, while only small changes in tyrosine phosphorylation can be detected at the protein level. Interestingly genistein also inhibits thyroid cell spreading on ECM components (Yap et al., 1994). Previous published data of Ridley and Hall (1994) clearly showed that the genistein inhibited the LPA- and bombesin-induced tyrosine phosphorylation of a number cellular proteins, one of which was formally shown to be pp125Fak. Whether these observations mean that the genistein-sensitive tyrosine kinase is essential for the tyrosine phosphorylation of cellular proteins induced by LPA, but not integrin-induced tyrosine phosphorylation remains to be established.

It is possible that rho plays a role in regulating the affinity of integrins for the matrix either by inducing inside-out signalling through the fibronectin receptor or by controlling the clustering of integrins. Microinjection of C3 transferase has been shown to induce rounding of cells (Paterson et al., 1990; Ridley and Hall, 1992), and also inhibits the inside-out signal inducing the interaction of LFA-1 with ICAM 1 and 3 in the activated lymphocyte (Tominaga et al., 1993). Moreover C3 transferase inhibits the α7β1-mediated clustering of activated platelets (Morii et al., 1992).

Dissection of the individual signalling events that control both the interaction of cells with the ECM and the formation of focal adhesions and actin stress fibres remain elusive. As well as increased protein tyrosine phosphorylation integrin activation also leads to elevation of intracellular pH and Ca^{2+} levels, and changes in gene expression and phospholipid metabolism (Juliano and Haskill, 1993). The rho proteins have been implicated in directly stimulating the activity of a number of signalling enzymes. Given that the generation of lipid second messengers may be involved in regulating proteins associated with cell adhesions, one interesting target of rho is PIP 5-kinase (Chong et al., 1994). This enzyme is involved in PIP2 synthesis, a lipid implicated in the regulation of actin polymerisation (Machesky and Pollard, 1993; Stossel, 1993) and in the regulation of a number of cytoskeletal proteins (Fukami et al., 1994; Weeke et al., 1996). Rho has
been shown to interact with the tail domain of a novel myosin like protein myr 5 which is also capable of associating with the cytoskeleton (Reinhard et al., 1995). This interaction may provide a degree of negative control upon rho once the cytoskeleton is assembled, and importantly provides a direct physical link between rho and the cytoskeleton.

In summary, the scrape loading and spreading experiments presented in this chapter clearly demonstrate that rho proteins lie downstream of both integrin-mediated signalling pathways and the heterotrimeric G-protein-mediated pathways regulating cell adhesion. The observation that integrin-mediated spreading is also affected by genistein implies that the genistein-sensitive kinase may be common to both pathways, but this requires further confirmation. A schematic representation of the possible integration of the integrin- and LPA-mediated pathways is shown in Fig. 4.12. There is mounting evidence that small GTP-binding proteins are involved in mediating adhesive responses to a variety of extracellular stimuli in a number of cell types. Despite this, the mechanisms through which they exert this control remains unclear. Identifying the direct downstream targets of the rho-like small GTP binding proteins, in the adhesion related signalling pathway, is required before the process can be understood in detail.
Figure 4.13. Schematic representation of the integration of the integrin and LPA-mediated signalling pathways. How signals from integrins are transduced to rho is unclear but it may involve a herbimycin A sensitive kinase. Signals from LPA to rho are transduced by a tyrphostin-sensitive kinase (Nobes et al., 1995). The events downstream of rho are transduced by a genistein-sensitive kinase, which in turn ultimately regulates the formation of focal adhesions through a staurosporine-sensitive kinase (Nobes and Hall, 1995a) and actin polymerisation through an unidentified factor possibly PIP 5-kinase (Chong et al., 1994).
Chapter Five

The role of protein kinase C in adhesion
5.1 Introduction

The previous chapters have provided evidence for a role for tyrosine kinases, tyrosine phosphatases and the small GTP-binding protein rho in regulating adhesive signals emanating from the interaction between cells and ECM proteins such as fibronectin, as well as soluble mitogenic factors. Although the tyrosine phosphorylation of certain proteins is associated with the formation of focal adhesions, a number of proteins that localise to the adhesion are not tyrosine phosphorylated. Two proteins that are not tyrosine phosphorylated upon LPA-stimulation of serum-starved Swiss 3T3 are vinculin and \( \beta_1 \)-integrin (Barry and Critchley, 1994). Furthermore \( \beta_1 \)-integrin and talin do not show tyrosine phosphorylation when cells are spread on fibronectin (Bockholt and Burridge, 1993). The activity of many proteins that do not show tyrosine phosphorylation may be regulated by phosphorylation on serine/threonine residues. Protein kinase C has been reported to play a role in adhesion regulation. Fibroblasts are unable to spread on the central cell-binding domain of fibronectin unless treated with the PKC-activating phorbol ester TPA (Woods and Couchman, 1992). They further inferred that PKC is required for cell spreading by using kinase inhibitors at concentrations that preferentially inhibit PKC. A role for PKC was also suggested by the recent demonstration that CHO cell spreading on fibronectin was blocked by the PKC inhibitor calphostin C. This study further demonstrated that TPA treatment increased spreading of CHO cells on fibronectin, and appeared to potentiate the tyrosine phosphorylation of p125Fak. The PKC activation observed by Vuori and Roussellet (1993) was shown to be a pre-ligand binding event though the ability of TPA to promote spreading has previously been shown to be a post receptor event (Danilov and Juliano, 1989). Pre-ligand binding implies that PKC is activated before the cells adhesive receptors are activated.

The role of PKC in regulating the assembly of focal adhesions has been investigated. Treatment of serum-starved Swiss 3T3 cells with TPA induced the formation of membrane ruffles (Ridley and Hall, 1992), implying that activation of PKC mediates an effect on the cytoskeleton. One PKC isoform, PKCa has previously been shown to localise to focal adhesions in rat embryo fibroblasts (REF52) (Jaken et al., 1989) providing further evidence for a possible role for PKC in adhesion regulation. However this has only been observed in one cell line with one individual antibody. In an attempt to extend these observations, the localisation of PKCa and other PKC isoforms in Swiss 3T3 fibroblasts was investigated.

Due to its co-localisation at focal adhesions, PKC has been implicated in regulating the recruitment of certain cytoskeletal proteins to focal adhesions. Two such proteins are vinculin and talin. Talin has been shown to be a substrate for PKC \textit{in vitro} and \textit{in vivo} in platelets (Lichfield and Ball, 1990a,b). Likewise vinculin is also a substrate for PKC \textit{in vitro} (Werth et al., 1983), and shows increased phosphorylation on serine/threonine
residues in cells treated with TPA (Werth and Pastan, 1984). The role of such phosphorylation events in regulating the function of these proteins has not been addressed further. Serum-starved Swiss 3T3 cells allow assessment of whether serine/threonine phosphorylation of cytoskeletal proteins is associated with the formation of focal adhesions. Here the phosphorylation of vinculin has been assessed. Early studies demonstrated that phosphorylated vinculin is associated with the Triton X-100 insoluble cytoskeleton (Geiger, 1982). Initially the distribution of phosphorylated vinculin between the Triton X-100 soluble and insoluble fractions was determined. The phosphorylation of vinculin was then assessed in serum-starved and stimulated cells to determine whether the phosphorylation of vinculin correlates with recruitment of the protein to focal adhesions.

Vinculin has been shown to be regulated by an intramolecular interaction between the N-terminal head and C-terminal tail (Johnson and Craig, 1994). This intramolecular association masks the binding sites for F-actin (Johnson and Craig, 1995a), talin (Johnson and Craig, 1994) and α-actinin (Kroemer et al., 1994). The in vivo studies on vinculin have been paralleled with in vitro analysis of factors regulating the head-tail interaction. The in vitro work was carried out in collaboration with Dr. John Weekes.

Results

5.2 PKC and adhesion formation in serum-starved Swiss 3T3 fibroblasts

5.2.1 TPA treatment of serum-starved Swiss 3T3 cells induces membrane ruffles and phosphotyrosine clusters

The simplest way to activate various PKC isoforms intracellularly is to stimulate the cells with TPA. Activation of PKC by TPA in serum-starved Swiss 3T3 cells has previously been shown to induce the formation of membrane ruffles similar to those generated upon activation of the small GTP-binding protein rac (Ridley et al., 1992). To further analyse the effects of PKC activation, serum-starved Swiss 3T3 cells (Fig. 5.1A,B) were treated with 100 nM TPA for 40 minutes (Fig. 5.1E,F) and then double stained for F-actin and phosphotyrosine to visualise any focal complexes formed. As a positive control, serum-starved cells were treated with either DMSO alone (Fig. 5.1A,B) or with 100 ng/ml LPA for 20 minutes in the presence of DMSO (Fig 5.1C,D). DMSO did not effect the serum-starved cells at the concentration used. Cells treated with 100 nM TPA (Fig. 5.1E,F) exhibited increased membrane ruffling, although some ruffling could be seen in a number of the DMSO control cells (Fig. 5.1A,B). Moreover the cells appeared to display small clusters of phosphotyrosine staining around the periphery of the cell. Although a few clusters of such staining could be seen in the untreated serum-starved cells these clusters appeared more randomly distributed than those seen in the TPA-stimulated cells. These results confirm that TPA treatment of serum-starved cells appears to stimulate the formation of membrane ruffles, but further suggests that small phosphotyrosine-containing focal clusters are also formed.
5.2.2 TPA inhibits LPA-mediated formation of focal adhesions and actin stress fibres

Given that PKC is required for the formation of focal adhesions (Woods and Couchman, 1992), it is reasonable to hypothesise that activation of PKC may well potentiate the LPA-induced assembly of focal adhesions. To test this hypothesis, cells were stimulated with 100 nM TPA and 100 ng/ml LPA simultaneously. Control serum-starved cells were treated with DMSO alone (Fig. 5.2A,B) or as a positive control with 100 ng/ml LPA in the presence of the appropriate concentration of DMSO (Fig. 5.2C,D). Cells treated with 100 nM TPA again exhibited a degree of membrane ruffling, with phosphotyrosine clusters formed around the periphery of the cell (arrowed, Fig. 5.2E,F), although in this case the phosphotyrosine panel is underexposed. Surprisingly stimulation of cells with both TPA and LPA did not result in the potentiation of the formation of focal adhesions and actin stress fibres. Rather the cells maintained a phenotype similar to cells treated with TPA alone, exhibiting only a few membrane ruffles with peripheral staining for phosphotyrosine clusters (Fig. 5.2G,H). This result suggests that LPA cannot induce the formation of focal adhesions or actin stress fibres when PKC is activated by TPA.

5.2.3 TPA induces the dissolution of focal adhesions and actin stress fibres in cultured Swiss 3T3 cells

The previous experiments suggest that a TPA-sensitive factor, presumed to be a PKC isoform, has an effect on the cytoskeleton of serum-starved cells. Moreover TPA treatment of serum-starved cells inhibits the LPA-mediated formation of focal adhesions (see also Ridley and Hall, 1994). Phorbol ester treatment of certain cell types has previously been reported to induce the loss of vinculin and α-actinin from focal adhesions and the dissolution of actin stress fibres (Meigs and Wang, 1986). To assess whether TPA treatment affects the cytoskeletal integrity of normal cultured Swiss 3T3 cells, TPA was applied at a concentration of 100 nM for 20 minutes. Control cells, treated with the appropriate amount of DMSO (Fig. 5.3A,B), contained actin stress fibres and strong vinculin-containing focal adhesions. Cells treated with 100 nM TPA however showed a loss in vinculin-containing focal adhesions and actin stress fibres (Fig. 5.3C,D), and instead displayed punctate actin staining and some membrane ruffles.

5.2.4 TPA induces the tyrosine phosphorylation of cellular proteins

As TPA appeared to generate the formation of small focal clusters of phosphotyrosine around the periphery of serum-starved cells, the ability of TPA to induce the tyrosine phosphorylation of cellular proteins was assessed. Serum-starved Swiss 3T3 cells were treated with either the appropriate amount of DMSO or 100 nM TPA for 5 and 20 minutes (Fig. 5.4A). As a positive control cells were also stimulated with LPA in the presence of DMSO. TPA clearly stimulates the tyrosine phosphorylation of proteins of around 116 kDa, possibly pp125Fak, and a diffuse band of protein at around 66 kDa, possibly paxillin.
Although this experiment is not quantitative, the TPA-generated tyrosine phosphorylation does not appear as intense as that induced by LPA, although proteins of a similar molecular mass were tyrosine phosphorylated upon stimulation with TPA and LPA. The tyrosine phosphorylation of proteins of 116 kDa and 66 kDa also occurred when cells were treated with 200 nM TPA over the same time points (Fig. 5.4C). To confirm whether one of the 116 kDa tyrosine phosphorylated proteins was pp125Fak, serum-starved cells were treated with 100 nM TPA for 20 minutes and the phosphotyrosine-containing proteins immunoprecipitated with an anti-phosphotyrosine antibody conjugated to agarose beads. The immunoprecipitate was then screened for the presence of pp125Fak by Western blotting with an antibody against pp125Fak (Fig. 5.4E). TPA treatment clearly leads to an increase in the amount of tyrosine phosphorylated pp125Fak. Western blotting of whole cell lysate with anti-paxillin antibody revealed that upon stimulation of cells with 100 nM TPA, paxillin becomes so heavily phosphorylated that its mobility is reduced by up to 10 kDa as estimated by SDS-PAGE (data not shown).

To assess how rapidly TPA could stimulate tyrosine phosphorylation of cellular proteins, serum-starved Swiss 3T3 cells were stimulated with 100 nM TPA for 30 seconds, 1, 2 and 5 minutes (Fig. 5.4F). A modest increase in tyrosine phosphorylation of proteins migrating at 116 kDa could be detected as early as 30 seconds after treatment. The results presented in Fig. 5.4 demonstrate that TPA, possibly by stimulating PKC isoforms, exerts a positive effect on the tyrosine phosphorylation of cellular proteins including pp125Fak.

To further correlate changes in cytoskeletal organisation with changes in tyrosine phosphorylation of cellular proteins, lysates of serum-starved cells stimulated with TPA, or TPA plus LPA, were analysed by Western blotting with an anti-phosphotyrosine antibody (Fig. 5.5A). Treatment of cells with TPA plus LPA did not result in higher levels of tyrosine phosphorylation than that observed in cells stimulated with TPA alone. Tyrosine phosphorylation of proteins of 116 and 66 kDa, as well as fainter bands of approximately 100 and 90 kDa could be seen in lysates of cells stimulated with both TPA and TPA/LPA. Although TPA inhibits LPA-induced cytoskeletal reorganisation in serum-starved cells, it induces the same pattern of cellular tyrosine phosphorylation observed upon stimulation of cells with LPA. These observations suggest that TPA inhibition of the LPA-induced assembly of focal adhesions occurs because TPA is initiating alternative signalling events, to those induced by LPA.

5.2.5 Inhibition of PKC rounds up serum-starved Swiss 3T3

The previous sections have established that TPA-mediated activation of PKC prevents the LPA-mediated formation of focal adhesions and actin stress fibres, while stimulating the tyrosine phosphorylation of a number of cellular proteins including pp125Fak. Although serum-starved cells exhibit little tyrosine phosphorylation, they still remain adherent
Figure 5.1. Treatment of Swiss 3T3 cells with 200 nM TPA stimulates the formation of membrane ruffles and phosphotyrosine containing focal adhesions. Serum-starved Swiss 3T3 cells were treated as follows. Control serum-starved cells treated with DMSO for 40 minutes (A,B). Control serum-stimulated cells treated with DMSO for 40 minutes and 100 ng/ml LPA for 20 minutes (C,D). Cells treated with 100 nM TPA for 40 minutes. Cells were double stained for F-actin (A,C,E) and for phosphotyrosine (B,D,F). Representative of more than two similar experiments. Bar represents 5 μm.
Figure 5.2. Pretreatment of Swiss 3T3 cells with 100 nM TPA inhibits the LPA-induced formation of actin stress fibres and focal adhesions. Serum-starved Swiss 3T3 cells were treated as follows. Control cells treated with solvent DMSO (A,B). Control cells treated with DMSO and 100 ng/ml LPA for 20 minutes (C,D). Cells treated with 100 nM TPA for 20 minutes (E,F). Cells treated with 100 nM TPA and 1 minute later with 100 ng/ml LPA for 20 minutes (G,H). Cells were double stained for F-actin (A,C,E,F) and phosphotyrosine (B,D,F,H). Arrows indicate the positions phosphotyrosine clusters. Representative of more than two similar experiments. Bar represents 5 μm.
Figure 5.3. TPA induces the dissolution of actin stress fibres and focal adhesions in Swiss 3T3 fibroblasts. Normal cultured subconfluent Swiss 3T3 cells were treated in the presence of serum with DMSO for 20 minutes (A,B), or 100 nM TPA for 20 minutes (C,D). Cells were double stained for F-actin (A,C) and vinculin (B,D). Representative of two similar experiments. Bar represents 5 μm.
Figure 5.4. **TPA induces the rapid tyrosine phosphorylation of cellular proteins including pp125Fak.** Serum-starved cells were stimulated with 100 nM TPA for 5 (5') and 20 (20') minutes, while control cells were treated with either DMSO (TPA 0) or DMSO plus 100 ng/ml LPA for 20 minutes (LPA 20'). Cells were lysed, boiled in protein sample loading buffer, equal amounts of lysate resolved by SDS-PAGE and analysed by Western blotting with an anti-phosphotyrosine antibody, PT66 (A). The gel was stripped and reprobed with an anti-vinculin antibody to indicate the loading in each lane (B). Molecular weight markers (kDa) are shown on the left hand side of each panel.

Serum-starved cells were also stimulated with 200 nM TPA for 5 (5') and 20 (20') minutes, while control cells were treated with DMSO (0). Cells were lysed and boiled into protein loading buffer, equal amounts resolved by SDS-PAGE and Western blotted with an anti-phosphotyrosine antibody, PT66 (C). The blot was then stripped and reprobed with an anti-vinculin antibody as an indication of the loading in each lane (D). Molecular weight markers (kDa) are represented on the left hand side of each panel.

Serum-starved cells were stimulated with 100 nM TPA for 20 minutes (20) while control cells were treated with DMSO for 20 minutes (0). Cells were lysed in RIPA buffer and the tyrosine phosphorylated proteins precipitated with an anti-phosphotyrosine antibody (PT66) conjugated to agarose beads. The immuneprecipitate was then boiled in protein loading buffer, resolved by SDS-PAGE and then probed for pp125Fak by Western blotting with an anti-pp125Fak antibody (E). Indicative of only one experiment. The molecular weight marker (kDa) is represented on the left of the panel.

The early effects of TPA stimulation were analysed by treating the cells with 100 nM TPA for 30 seconds (30") and 1 (1'), 2 (2'), and 5 (5') minutes. The cells were boiled into protein loading buffer, equal amounts resolved by SDS-PAGE and then Western blotted with an anti-phosphotyrosine (PT66) antibody (F). The blot was then stripped and probed with an anti-vinculin antibody to determine the loading in each lane (G). Indicative of only one experiment. Molecular weight markers (kDa) are represented on the left hand side of each panel.
100 nM TPA

A  Phosphotyrosine

B  Vinculin

Phosphotyrosine immunoprecipitation (100 nM TPA)

E  pp125Fak

Early effects of TPA stimulation

F  Phosphotyrosine

G  Vinculin
Figure 5.5. LPA does not enhance TPA induced tyrosine phosphorylation. Serum-starved cells were treated with either DMSO (0), 100 nM TPA for 20 minutes (TPA), and 100 nM TPA plus 100 ng/ml LPA for 20 minutes (TPA + LPA). The cells were boiled into protein loading buffer, the lysates separated by SDS-PAGE and Western blotted with an anti-phosphotyrosine (PT66) antibody (A). The blot was stripped and reprobed with an anti-vinculin antibody to determine the loading in each lane (B). Indicative of only one experiment. Molecular weight markers (kDa) are represented on the left hand side of each panel.
through an RGD-dependent interaction with the ECM (Chapter 4). Given that PKC is required for cells to spread on fibronectin (Woods and Couchman, 1992; Vuori and Ruoslahti, 1993), it is possible that PKC activity is required to maintain the spread phenotype. The effect of inhibiting PKC on the organisation of actin stress fibres and focal adhesions was initially studied using the cell-permeable inhibitor bisindolylmaleimide (Toullec et al., 1991). Although this inhibitor was seen to reduce the LPA-mediated stress fibre formation, aggregates of inhibitor were seen to lie in the cytoplasm around the nuclear region (data not shown). This implies that once in the intracellular environment, the inhibitor may be insoluble or be directed to a particular subcellular location. However, bisindolylmaleimide has been shown to reduce the LPA-mediated tyrosine phosphorylation of the 110-130 kDa band, and specifically pp125Fak, in quiescent but not serum-starved Swiss 3T3 cells (Seufferlein and Rozengurt, 1994).

The effects of the PKC inhibitor calphostin C on serum-starved cells was assessed. Cells were incubated with 0.25, 0.5, 1.0 and 2.0 μM calphostin C for one hour in the presence of a fluorescent light, required to photo-activate the compound (Kobayashi et al., 1989; Bruns et al., 1991). Calphostin C treatment induced rounding of serum-starved cells within one hour (Fig. 5.6). As the cells had to be placed in a tissue culture incubator for a period of time the fluorescent light was placed in a sealed clear bag on the shelf above. It is unlikely that illumination in this manner is sufficient for full activation, as when the same experiment is performed on a CO2 stage under full fluorescent light the effect of the inhibitor on serum-starved cells was far more rapid (Anne Ridley, personal communication). Despite this, the result implies that the serum-starved cells remain spread by virtue of a calphostin C-inhibitable kinase activity, which at the concentrations used is likely to be one of a number of PKC isoforms (Kobayashi et al., 1989; Bruns et al., 1991).

5.2.6 LPA does not prevent calphostin C-induced rounding
To determine whether LPA-stimulation of cells could inhibit the calphostin C-induced rounding, cells were preincubated with 1 μM calphostin C for 0, 15, 30 and 45 minutes, then treated with LPA for a further 15 minutes before being fixed and stained for F-actin. Cells treated with calphostin C alone for as little as 15 minutes (Fig. 5.7C) possessed a number of short actin filaments distinct from LPA-stimulated actin stress fibres (Fig. 5.7B). After 30 minutes with calphostin C, these short filaments were still evident (Fig. 5.7G) but the cell had contracted, and the cell body was smaller than that of serum-starved (Fig. 5.7A,E,I,M) or LPA-stimulated cells (Fig. 5.7B,F,J,N). After 45 minutes, this contraction was more pronounced with the cells beginning to display retraction fibres, but short actin filaments were still visible (Fig. 5.7K). Once the cells had been exposed to calphostin C for 60 minutes, the short actin filaments were no longer visible, and instead cells appeared rounded. Many cells exhibited retraction fibres and stained brightly with phalloidin (Fig. 5.7O). Addition of LPA at any time point had no dramatic effect on the
phenotype of the cells (Fig. 5.7D,H,L,P). Most importantly calphostin C still induced
detachment of cells in the presence of LPA (Fig. 5.7O,P). Indeed the retraction may even
be slightly enhanced by the addition of LPA. The data suggests that LPA is unable to
induce the formation of actin stress fibres and normal focal adhesions in the presence of
calphostin C.

The nature of the actin filaments induced by calphostin C treatment were investigated
further. Serum-starved Swiss 3T3 cells were treated with 1 μM calphostin C for 20
minutes. As a reference, control serum-starved cells were stimulated with LPA for the
same period (Fig. 5.8 E,F). Cells were double stained for actin and either vinculin or
phosphotyrosine. The actin filaments formed in cells treated with calphostin C (Fig.
5.8E,G) are clearly distinct from the actin stress fibres formed in cells treated with LPA
(Fig. 5.8C). LPA-induced fibres are generally organised in long straight fibres running in
parallel from the focal adhesion at the cell periphery through the body of the cell
(arrowed). The fibres induced by calphostin C were short, disorganised and appeared
randomly distributed throughout the cell body (arrowed). Calphostin C treated cells
stained less intensely for F-actin than cells stimulated with LPA. The difference between
the actin filaments is also reflected in the nature of the focal complexes formed. Large
vinculin-containing focal adhesions can be clearly seen after 20 minutes in cells stimulated
with LPA (Fig. 5.8D). However in the calphostin C treated cells the vinculin-containing
focal complexes are much smaller and stain faintly with the anti-vinculin antibody (Fig.
5.8F). The calphostin C-induced phosphotyrosine-containing clusters are also much
smaller than found in LPA-stimulated cells (Fig. 5.8H). This data implies that calphostin
C treatment of serum-starved Swiss 3T3 stimulates the formation of short actin filaments
associated with small clusters of focal adhesion-associated proteins, and is distinct from
the response induced by LPA treatment.

5.3 Localisation of PKC isoforms to focal adhesions in Swiss 3T3 fibroblasts

5.3.1 PKCα cannot be detected in focal adhesions of growing subconfluent Swiss 3T3
cells

Part of the limited evidence that suggests a role for PKC in modulating cell adhesion is the
localisation of PKCα to focal adhesions in REF52 cells (Jaken et al., 1989). To
characterise the time course of recruitment of PKCα to focal adhesions, normal
subconfluent Swiss 3T3 fibroblasts were examined by immunofluorescence with a
antibody which recognizes the classical PKC isoforms α, βI and βII. This antibody
recognises PKCα in immunohistochemical analysis (Fig. 5.9A), but reveals no focal
staining in Swiss 3T3 cells. The cells were double-stained with a polyclonal antibody
against talin to show that the integrity of the focal adhesions was not destroyed by the
fixation procedure (Fig. 5.9B). PKCα did not co-localise with focal adhesions, revealed
with the polyclonal talin antibody, although PKCα was expressed in the Swiss 3T3 cells
Figure 5.6. Calphostin C induces rounding of serum-starved Swiss 3T3 cells. Serum-starved cells were treated with a range of concentrations of the PKC inhibitor calphostin C and illuminated by a fluorescent light placed on the shelf above in the tissue culture incubator. Cells were fixed and stained with crystal violet after 1 hour. Control cells were treated with the appropriate concentration of solvent DMSO for 1 hour (A,C,E,G). Representative of more than two similar experiments. Cells were treated with 0.25 μM (B), 0.5 μM (D), 1.0 μM (F) and 2.0 μM (H) for 1 hour. Bar represents 50 μM.
Figure 5.7. LPA does not prevent calphostin C-induced cell rounding. Serum-starved Swiss 3T3 cells were treated with 1 μM calphostin C, and after varying lengths of incubation stimulated with LPA for 15 minutes. Control serum-starved cells were treated with DMSO for the appropriate length of time (A,E,I,M) or stimulated with LPA for 15 minutes after preincubation for 0 minutes (B), 15 minutes (F), 30 minutes (J) and 45 minutes (N). Cells were also treated with 1 μM calphostin C alone for 15 minutes (C), 30 minutes (G), 45 minutes (K) and 60 minutes (O), or with LPA added for the final 15 minutes of the incubation period (D,H,L,P respectively). Representative of two similar experiments. Cells were fixed and stained for F-actin. Bar represents 5 μm.
Figure 5.8. Calphostin C induces the formation of short actin filaments. Serum-starved cells were treated with 1 μM calphostin C for 20 minutes. Control serum-starved cells were treated with the appropriate amount of solvent DMSO (A,B) or stimulated with 100 ng/ml LPA in the presence of DMSO for 20 minutes (C,D). Cells were also treated with 1 μM calphostin C for 20 minutes (E,F,G,H). Cells were fixed and double stained for F-actin (A,C,E,G) and either vinculin (B,D,F) or phosphotyrosine (H). Bar represents 5 μM.
This result suggests that PKCα may not be localised to focal adhesions in Swiss 3T3 fibroblasts.

5.3.2 An antibody raised against PKCδ stains focal adhesions
Swiss 3T3 fibroblasts are known to express four PKC isoforms α, δ, ε and ζ (Olivier and Parker, 1994) and the possibility that one or other of these PKC isoforms localise to focal adhesions was therefore assessed. A monoclonal antibody raised against PKCδ has been observed to stain structures in fibroblasts reminiscent of focal adhesions (marketed by Transduction Laboratories). This antibody reacts with an 80 kDa protein within Swiss 3T3 cell lysates, the same molecular weight as PKCδ, but also a band at approximately 50 kDa (Fig. 5.10). A polyclonal antibody against rat-PKCδ (courtesy Peter Parker), did not recognise mouse PKCδ migrating at 80 kDa, but did react with the protein migrating at 50 kDa. This suggests that this 50 kDa species is a non-specific band recognised by either both primary or both secondary antibodies. To assess whether PKCδ localised to newly forming focal adhesions, serum-starved Swiss 3T3 cells (Fig. 5.10A,B) were stimulated with LPA for 20 minutes (Fig. 5.10C,D) and stained for actin and PKCδ with the Transduction Laboratory antibody. The PKCδ antibody clearly reacts with a protein that localises to the ends of actin stress fibres within 20 minutes of stimulation with LPA (Fig. 5.10D), while in the serum-starved cells it appeared to stain the nucleus and perinuclear region, as well as the plasma membrane (Fig. 5.10B). The time course of the appearance of this focal staining with the anti-PKCδ antibody was then investigated. Serum-starved Swiss 3T3 were stimulated with LPA for 2, 5 and 10 minutes, and double stained for actin and PKCδ (Fig. 5.11). Unlike the recruitment of vinculin and paxillin to focal adhesions, the PKCδ antibody did not reveal any such localisation until 5 minutes after stimulation, coincident with the appearance of actin stress fibres (Fig. 5.11E,F). Before the appearance of these focal adhesion like structures, the the most prominent staining within the cells is around the nuclear region (Fig. 5.11B,D), which is still visible, but reduced after the focal staining appears (Fig. 5.11F,H). The time course reveals that the anti-PKCδ antibody only exhibits focal staining once actin stress fibres are formed. Unlike antibodies against other focal adhesion proteins, the PKCδ antibody does not recognise the small focal complexes formed before stress fibres are visible.

5.3.3 Confirmation of the cellular localisation of PKCδ
To further investigate the possibility that PKCδ is localised to the ends of actin filaments, rat PKCδ cDNA, was transiently expressed in monkey Cos cells. Cos cells were chosen as they express little or no endogenous PKCδ as determined by Western blotting with an anti-rat PKCδ polyclonal antibody (Dr. Peter Parker, personal communication). Cos cells were transiently transfected using the calcium phosphate method as described in Materials and Methods (Chapter 2), with 14 and 18 μg PKCδ cDNA cloned into the high level expression vector pMT (kindly supplied by Dr. Peter Parker, ICRF, London). Expression was allowed
to proceed for 3 days at which point the cells were taken into SDS-PAGE loading buffer to analyse for expression of the protein by Western blotting. Other cells were fixed and stained for analysis of the proteins localisation by immunofluorescence. Expressed PKCδ was detected using both the monoclonal antibody (Transduction Laboratories) and a polyclonal antibody against rat PKCδ (supplied by Dr. Peter Parker). Expression of the rat PKCδ could be detected by both antibodies, the protein migrating at approximately 75-80 kDa (Fig. 5.13A,B). Interestingly the monoclonal antibody recognised a protein of about 85 kDa in the untransfected Cos cells, not recognised by the polyclonal antibody raised against rat PKCδ (Fig. 5.13A). This may be monkey PKCδ which does not cross react with the polyclonal antibody or alternatively another protein with which the monoclonal antibody cross-reacts. The bands migrating at 35 kDa and between 40 kDa and 66 kDa are likely to be breakdown products of PKCδ, primarily detected by the polyclonal antibody, as it recognises more than one epitope on PKCδ (Fig. 5.13B). Both antibodies recognize the rat PKCδ, which appears to be expressed at high levels.

5.3.4 Other approaches to investigate the localisation of PKCδ

The cellular distribution of the expressed rat PKCδ was examined by immunofluorescence. Cells were either fixed and extracted with 0.2% Triton or extracted with MES buffer and then fixed prior to staining, as described in Materials and Methods (Chapter 2). When cells were extracted prior to fixation no rat PKCδ could be detected with either antibody (data not shown). When cells were fixed prior to extraction, expressed protein could be detected with both monoclonal (Fig. 5.12A,B) and polyclonal (Fig. 5.12C,D,E,F). However specific localisation of the expressed rat PKCδ could not be detected, the protein being distributed throughout the cytoplasm. One problem was that very few of the Cos cells examined that expressed rat PKCδ possessed actin filaments (Fig. 5.12A,C). This was consistent throughout the population of cells, not restricted to only those cells expressing PKCδ. Background staining of non-transfected cells could not be detected with either antibody. Although rat PKCδ was expressed and recognised by both antibodies in Cos cells this approach did not allow identification of the cellular localisation of rat PKCδ.

The cytosolic localisation of PKCδ observed using the transient expression method may have been due to a number of factors. The high level of expression of the protein may mask the true distribution of PKCδ in cells. The Cos cells used possessed few, actin stress fibres and therefore only a small number of focal adhesions. Alternatively the protein may be unable to localise properly in Cos cells. The cellular localisation of PKCδ may be successfully studied by introducing epitope tagged PKCδ into cell lines known to express the protein endogenously. To this end, two constructs were made, a GST-PKCδ fusion protein and a myc tagged fusion protein. The rat PKCδ cDNA lacking the initiating methionine was first cloned into the pGEX-2T vector for expression as a fusion protein. The expressed GST-PKCδ fusion protein could then be microinjected, into Swiss or Balb/c
3T3 fibroblasts and the protein localised by immunofluorescence using a polyclonal anti-GST antibody to detect the injected rat PKCð over endogenous the mouse protein. Unfortunately although PKCð was successfully expressed as a full length fusion protein it was targeted to inclusion bodies, and as an insoluble protein was of no use for microinjection.

The second approach taken was to epitope tag the rat PKCð cDNA with a conserved 12 amino acid epitope of the e-myc protein recognised by a monoclonal antibody known as 9E10 (Evan et al., 1985). Such an approach has been successfully used to study the insulin-induced translocation of the GLUT4 glucose transporter from the intracellular pool to the membrane (Kanai et al., 1993). A vector containing the myc tag epitope tag, 5’ of a multiple cloning site was obtained from Richard Triesman (ICRF, London). A PKCð cDNA fragment flanked with specific restriction enzyme sites was generated by PCR. This allowed the cDNA to be cloned in frame with the epitope tag. This produced a PKCð cDNA tagged at the 5′-end with the myc epitope. The cDNA encoding the fusion protein was then inserted into two mammalian expression vectors, the fragment again generated by PCR flanked with two restriction sites. The fragment was inserted into pCDNA-3 (Invitrogen) which allows constitutive expression from the cytomegalovirus promoter and into pMAMneo BLUE (Clontech), which allows dexamethasone-inducible expression of the protein of interest from the mouse mammary tumour virus promoter. A full description of the cloning strategy is included in Materials and Methods (chapter 2). Balb/c 3T3 cells were transfected with both constructs using the calcium phosphate method and stable cell lines containing both plasmids established by G418 selection.

Clones were initially analysed for expression of myc tagged PKCð by immunofluorescence, though no staining significantly different from that of the negative controls was observed (data not shown). As a result cell lysates were prepared from all clones obtained and analysed by Western blotting with the 9E10 antibody to determine whether any of the clones were expressing the tagged fusion at levels high enough to allow analysis by immunofluorescence (Fig. 5.14). As a postive control, lysate containing a 9E10 epitope tagged Flp recombinase expressed in Cos cells was also included on the blots (kind gift from Paul Walker, University of Leicester). Although the positive control can be clearly seen at approximately 50 kDa (arrowed), no tagged PKCð can be seen at around 80-85 kDa (arrowed) even though the blot has been exposed such that non-specific bands (example 116 kDa) are evident. Such non-specific bands are commonly seen when blots are detected with this antibody. This indicates that despite isolation of a number of G418-resistant clones, none is expressing the myc tagged PKCð at high enough levels to allow detection by Western blotting.

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Figure 5.9. Expression and cellular localisation of PKCα in Swiss 3T3 cells. Normal subconfluent Swiss 3T3 cells in serum were examined for the expression of PKCα. A-Cells were methanol-fixed and doubly stained with an anti-PKCα antibody (A) and with a polyclonal anti-talin antibody (B). Representative of two experiments. Bar represents approximately 3 μm. B- Similar cells were taken into protein loading buffer, an aliquot of the lysate resolved by SDS-PAGE and Western blotted with the anti-PKCα monoclonal using an alkaline phosphatase detection system. Molecular weight markers (kDa) are represented on the left hand side of panel B.
Figure 5.10. An antibody raised against human PKCδ stains the ends of actin filaments extending from newly formed focal adhesions. (Top) Serum starved Swiss 3T3 cells (A,B) were stimulated with 100 ng/ml LPA for 20 minutes (C,D). The cells were double stained for F-actin (A,C) and PKCδ (B,D), with the monoclonal antibody from Transduction Laboratories. Bar represents 10 μm. (Bottom) Western blot of Swiss 3T3 whole cell lysate with the polyclonal and monoclonal PKCδ antibodies as indicated. The volume of lysate loaded in the left hand lane was double that loaded in the right hand lane of each blot. Molecular weight markers (kDa) are shown on the left of each panel.
Figure 5.11. Time course of recruitment of PKCδ to focal adhesions. Serum-starved cells (A,B) were stimulated for 2 minutes (C,D), 5 minutes (E,F) and 10 minutes (G,H) with 100 ng/ml LPA. Cells were fixed and double stained for F-actin and with the anti-PKCδ monoclonal antibody. Bar represents 3 μm.
Figure 5.12. (Top) Transfection of monkey Cos cells with a rat PKC\(\delta\) cDNA expression construct (pMT). Monkey Cos cells were transfected with a total of 14 or 18 \(\mu\)g per dish of the pMT-rat PKC\(\delta\) using the calcium phosphate transient transfection method and analysed after 3 days. The cells were fixed, Triton extracted, and double stained for F-actin (A,C,E) and PKC\(\delta\) with the monoclonal anti-human antibody (B) or the polyclonal PKC\(\delta\) antibody (D,F). Representative of three similar experiments. Bar represents 5 \(\mu\)m

Figure 5.13. (Bottom) Expression of the rat PKC\(\delta\) cDNA in monkey Cos cells. Cos cells transiently transfected with either PKC\(\delta\)-pMT and mock transfected cells (0) were taken into protein loading buffer after 3 days, equal amounts of lysate resolved by SDS-PAGE and then Western blotted with either the monoclonal anti-human PKC\(\delta\) antibody (Transduction Laboratories) (A) or the polyclonal anti-PKC\(\delta\) antibody. Molecular weight markers (kDa) are represented on the left hand side of both panels.
Figure 5.14. Expression of myc-tagged PKCδ fusion protein in Balb/c 3T3 cells. Lysates were prepared from G418-resistant clones of Balb/c 3T3 cells transfected with both the pCDNA3 and pMAMneo blue constructs, and from a number of untransfected and uninduced negative controls. Equal amounts of lysate were resolved by SDS-PAGE and Western blotted with the 9E10 anti-myc antibody. The expected position of the expressed tagged PKCδ is arrowed. The identity of each sample is indicated, as is the presence of the positive control (+VE, bottom of this lane is asterixed); PC represents clones transfected with pCDNA-3; PM represents clones transfected with pMAM-NEO; DEX represents clones treated with DEX and ETOH; clones treated with ethanol. Molecular weight markers (kDa) are indicated on the left hand side of each panel.
5.4 Regulation of the cytoskeletal protein vinculin

5.4.1 Vinculin is a phosphoprotein

Early work by Sefton et al. (1981) and Geiger (1982) suggested that vinculin was a phosphoprotein, normally phosphorylated mainly on serine residues and some threonine residues, but also on tyrosine by v-src in RSV transformed cells. Subsequent work by Werth and Pastan (1983; 1984) indicated that vinculin was an in vitro substrate for PKC and that in vivo phosphorylation was increased upon treatment of Swiss 3T3 and chick embryo fibroblasts with calcium and phorbol esters. This work was therefore repeated to assess whether vinculin is indeed a phosphoprotein. Cultured Swiss 3T3 cells were labelled with $^{32}$P orthophosphate in the presence of serum, stimulated with 100 nM TPA for 20 minutes then lysed into RIPA buffer. The labelled vinculin was immunoprecipitated with an anti-human vinculin antibody (Fig. 5.15A). Although vinculin seems to be weakly labelled in normal cultured cells, no more phosphate was seen to be incorporated upon TPA stimulation. A small aliquot of the labelled lysate was analysed SDS-PAGE revealing that numerous cellular proteins were phosphorylated during the labelling procedure (Fig. 5.15B). The results confirm that vinculin is indeed a phosphoprotein but that the phosphorylation of the protein is not increased upon activation of PKC isoforms with TPA in Swiss 3T3 cells.

5.4.2 Vinculin does not show increased phosphorylation upon stimulation with LPA

To determine whether the cells were responding to LPA under the conditions of the labelling procedure, control experiments were performed in phosphate-free media with the appropriate concentration of unlabelled orthophosphate. This was initially investigated by immunofluorescence. Cells plated on coverslips were treated as for cells undergoing $^{32}$P orthophosphate labelling, serum-starved and then stimulated with LPA for 20 minutes. The cells were then double stained for actin and phosphotyrosine. Serum-starved cells in low phosphate conditions (Fig 5.16E,F) resembled the control serum-starved cell (Fig. 5.16A,B), neither containing actin stress fibres, nor phosphotyrosine-containing focal adhesions. Upon stimulation with LPA, the cells in low phosphate conditions formed both phosphotyrosine-containing focal adhesions and actin stress fibres (Fig. 5.16G,H). The numbers of actin stress fibres formed appeared slightly lower in the low phosphate conditions (Fig. 5.16G) compared to control cells (Fig. 5.16C) although the formation of phosphotyrosine-containing focal adhesions was unaffected. The change in LPA-mediated cellular protein tyrosine phosphorylation in cells starved under low phosphate conditions was also investigated by Western blotting with the anti-phosphotyrosine antibody PY20 (Fig. 5.17A). The cells responded normally to LPA exhibiting increased tyrosine phosphorylation of proteins migrating just above the 66 kDa marker, 100 kDa, complex of proteins between 116 and 130 kDa and one at 210 kDa. The blot was stripped and reprobed for vinculin (Fig. 5.17B), revealing that slightly less protein was loaded in the serum-starved lane. However the data confirms that even in low phosphate conditions the
serum-starved cells respond normally to LPA with respect to the formation of actin stress fibres and focal adhesions, and tyrosine phosphorylation of cellular proteins.

Having established that vinculin was a phosphoprotein, the serum-starved Swiss 3T3 cells were utilised to assess whether vinculin was phosphorylated upon the formation of the focal adhesions. Cells were deprived of phosphate in serum-free media for 45 minutes, the media changed, and then the cells starved of serum for 16 hours in the presence of 100 μCi/ml 32P orthophosphoric acid. After 16 hours the cells were stimulated with LPA for 20 minutes. The incorporation of 32P-phosphate into vinculin upon LPA-stimulation of serum-starved cells was determined by immuneprecipitation. Very little vinculin was phosphorylated in the serum-starved cells and no increased incorporation was detectable upon LPA-stimulation (Fig. 5.18A). Analysis of a small aliquot of each of the labelled cell lysate revealed that numerous cellular proteins became labelled under the conditions of the experiment (Fig. 5.18B).

5.4.3 Phosphorylated vinculin is predominantly in the cytosolic fraction

Work by Geiger (1982) has shown that phosphorylated vinculin in CEF cells was associated predominantly with the Triton X-100 insoluble cytoskeleton, therefore the distribution of phosphorylated vinculin in Swiss 3T3 cells was determined. One further possibility that required consideration was that preparing the cell lysates for immuneprecipitation in RIPA buffer may not have solubilised the phosphorylated vinculin. To take this possibility into account an alternative extraction procedure was devised.

To obtain a Triton X-100 insoluble cytoskeletal fraction and a Triton X-100 soluble cytosolic fraction, Swiss 3T3 cells were fractionated by washing the monolayer with a Triton X-100 (0.2% or 1%) solution. To determine the distribution of vinculin between these fractions, Swiss 3T3 were 35S-methionine labelled and extracted under different conditions to achieve a crude cellular fractionation (Fig. 5.19). Cells were incubated on ice, with 0.2% Triton X-100 for 2 and 4 minutes, and with a 1% Triton X-100 solution for 2 minutes, and then the extraction buffer removed. The Triton X-100 soluble lysate was adjusted to the same detergent concentrated and composition as RIPA buffer normally used for immuneprecipitation.

The remaining Triton X-100 insoluble cytoskeleton was then scraped into a 1% SDS buffer, heated at 80°C for 2 minutes to disrupt protein interactions, and ensure that all the cytoskeletal associated vinculin was in solution. The lysate was cooled on ice and then the SDS concentration diluted to give a lysate of the same detergent concentration as RIPA buffer. The 35S-methionine labelled vinculin was immuneprecipitated from whole and fractionated cell lysates (Fig. 5.19). Under the various extraction stringencies it appears that between 30-40% of the vinculin was associated with the cytoskeleton in Swiss 3T3
Figure 5.15. Vinculin is a phosphoprotein. Normal subconfluent Swiss 3T3 cells were labelled for 16 hours with $^{32}$P orthophosphoric acid in phosphate free DMEM (10% dialysed FCS) at a concentration of 100 μCi/ml. Cells were then treated with either solvent DMSO (Cult) or with 100 nM TPA (TPA) for 20 minutes and then lysed into RIPA buffer. Vinculin was immuneprecipitated with the anti-human vinculin antibody from both lysates, the precipitate boiled into protein loading buffer and resolved by SDS-PAGE. The phosphorylated vinculin was visualised by autoradiography (A). To show that many proteins in the lysate became labelled under the conditions of the experiment small aliquots of each lysate were likewise resolved by SDS-PAGE and visualised by autoradiography (B). Representative of two experiments. Molecular weight marker (kDa) is represented on the left hand side of panel (A).
Figure 5.16. Serum-starved Swiss 3T3 cells respond normally to LPA under low phosphate conditions. Normal serum-starved cells and cells serum-starved in phosphate free media (spiked with cold orthophosphoric acid at a concentration equivalent to 100 μCi/ml 32P orthophosphoric acid), and stimulated with 100 ng/ml LPA for 20 minutes. Normal serum-starved cells (A,B). Normal cells stimulated with 100 ng/ml LPA for 20 minutes (C,D). Cells serum-starved under low phosphate conditions (E,F). Cells starved under low phosphate conditions stimulated with 100 ng/ml LPA for 20 minutes (G,H). Representative of two experiments. Cells were fixed, permeabilised and double stained for F-actin (A,C,E,G) and phosphotyrosine (B,D,F,H). Bar represents 5 μm.
Figure 5.17. Swiss 3T3 cells serum-starved under low phosphate conditions show cellular protein tyrosine phosphorylation in response to LPA. Confluent Swiss 3T3 cells were starved under low phosphate (labelling) conditions (0) and then stimulated with 100 ng/ml LPA for 20 minutes (20). The cells were then lysed into protein loading buffer, equal amounts of lysate resolved by SDS-PAGE and Western blotted with an anti-phosphotyrosine antibody (PY20) (A). The blot was then stripped and reprobed with the anti-vinculin antibody to determine the loading in each lane (B). Molecular weight markers (kDa) are represented on the left hand side of panel (A).
Figure 5.18. Vinculin does not show increased phosphorylation upon stimulation of starved cells with LPA. Confluent serum-starving Swiss 3T3 cells were labelled for 16 hours with \( ^{32}\)P orthophosphoric acid in phosphate free DMEM at a concentration of 100 \( \mu \)Ci/ml. Starved cells were then either untreated (0) or stimulated with 100 ng/ml LPA (LPA) for 20 minutes, and then lysed into RIPA buffer. Vinculin was then immuneprecipitated with the anti-human vinculin antibody from both lysates, the precipitate boiled into protein loading buffer, resolved by SDS-PAGE, and the phosphorylated vinculin visualised by autoradiography (A). To show that protein in the lysate became labelled under the conditions of the experiment, small aliquots of each lysate were resolved by SDS-PAGE and visualised by autoradiography (B). Representative of more than two similar experiments. Molecular weight marker is represented on the left hand side of panel (A).
cells, as determined by phosphorimage analysis (data not shown). Increasing the
stringency of extraction did not increase the amount of vinculin taken into the Triton X-
100 fraction. The amount of vinculin in the cytoskeletal fraction observed with this
technique is higher than that originally reported by Geiger (1982) who found that between
10-15% of the cellular vinculin was in the cytoskeleton. This discrepancy may be due to a
number of factors including the difference in cell type and the nature of the fractionation
procedures used.

The same procedure was used to fractionate cells labelled with $^{32}$P orthophosphate in the
presence of serum. Cells were extracted with 0.2% and 1% Triton X-100 for 2 minutes
and the vinculin precipitated from the Triton X-100 insoluble, the Triton X-100 soluble
and whole cell lysates. The fractionation reveals that the $^{32}$P-labelled vinculin is
predominantly associated with the fraction extracted by the Triton X-100 buffers (Fig.
5.20A). The labelling of vinculin in this cell line is very low especially when considering
that the vinculin immunoprecipitated from the lysates could be clearly seen when the gel
was stained with Coomassie blue (Fig. 5.20B). The data indicates that phosphorylation of
vinculin is not associated with the recruitment of the protein to the cytoskeleton.

5.4.4 Recruitment of vinculin to the cytoskeleton upon the formation of focal
adhesions
The change in subcellular distribution of vinculin between the Triton X-100 soluble and
insoluble fraction upon formation of focal adhesions was investigated. Initially the
fractionation was performed on serum-starved and LPA-stimulated Swiss 3T3 cells (Fig.
5.21). The fractionation revealed that there was no obvious difference in the distribution of
vinculin between the Triton X-100 soluble and insoluble fractions. After two minutes
extraction there actually appears to be more vinculin associated with the cytoskeleton of
the starved cells. The data suggests that upon serum deprivation vinculin remains
associated with the cytoskeleton under conditions where there are no detectable focal
adhesions. More experiments are required to obtain a more precise picture of the change in
vinculin distribution, which would allow quantitation of the total vinculin distribution.

The same experiment was performed using cells in suspension versus cells spread on
fibronectin for 60 minutes (Fig. 5.22). Serum-starved cells were trypsinised, and once in
suspension the action of the trypsin inhibited with trypsin inhibitor. The cells were then
either kept in suspension in a polypropylene tube by occasional agitation, or spread on
tissue culture dishes coated with 50 μg/ml fibronectin, for 60 minutes in serum-free media.
The cells were extracted with 0.2% Triton X-100 buffer for 2 and 4 minutes. The cells
maintained in suspension were first spun down at 6500 rpm for 30 seconds and then gently
resuspended in the Triton X-100 buffer. The insoluble fraction pelleted by spinning at
13000 rpm, 30 seconds before the end of the incubation. Cells maintained in suspension
still contained vinculin in the Triton X-100 insoluble fraction. The presence of vinculin in the Triton X-100 insoluble fraction of starved cells maintained in suspension, under conditions of increasing stringency implies that it may be constitutively associated with a Triton X-100 insoluble fraction in fibroblasts. This is consistent with the vinculin distribution observed in serum-starved adherent cells (Fig. 5.21), with vinculin in the insoluble fraction. The data obtained from the fractionation experiments suggests that in Swiss 3T3 fibroblasts vinculin is constitutively associated with the Triton X-100 insoluble fraction even under conditions where the cells do not possess detectable focal adhesions or actin stress fibres.

The association of the phosphotyrosine-containing proteins with the Triton X-100 insoluble cytoskeleton were studied using a similar Triton X-100 fractionation technique. It was of interest to know whether the tyrosine phosphorylated proteins become incorporated into the Triton X-100 insoluble cytoskeleton upon stimulation of serum-starved Swiss 3T3 cells. The levels of vinculin and phosphotyrosine in whole cell lysates and in the insoluble fraction of cells extracted with a 0.2% Triton X-100 solution for 2 minutes were compared. Although a similar proportion of the vinculin was resistant to Triton X-100 extraction even in the serum-starved cells (Fig. 5.23A), the phosphotyrosine containing bands at 116 kDa and 66 kDa were removed upon incubation with the Triton X-100 buffer (Fig. 5.23B). These phosphotyrosine proteins corresponded to the molecular weights of pp125Fak and paxillin, previously shown to become tyrosine phosphorylated upon LPA stimulation of serum-starved cells. The experiment was repeated and the whole cell lysate and Triton X-100 cytoskeleton probing for the presence of pp125Fak and paxillin. This revealed that both proteins were indeed removed by Triton X-100 extraction (Fig. 5.23C,D). This suggests that both paxillin and pp125Fak may not be integral components of the actin cytoskeleton.

To confirm these observations, the phosphotyrosine immunoprecipitates of fractions from cultured, serum-starved and LPA-stimulated cells were analysed for pp125Fak and paxillin by Western blotting (Fig. 5.24). The majority of the pp125Fak was detected in the cytosolic fraction of the LPA-stimulated cells, with only a trace associated with the cytoskeleton. A similar distribution was observed when the precipitate was also probed for paxillin. The result again suggests that tyrosine phosphorylated pp125Fak is not an integral part of the actin cytoskeleton.
Figure 5.19. Distribution of vinculin between the soluble and cytoskeletal fractions. To determine the distribution of vinculin between the Triton X-100 soluble and cytoskeletal fractions, $^{35}$S-methionine labelled cells were extracted with a 0.2% or 1.0% Triton buffer for 2 or 4 minutes, as indicated. Vinculin was immuneprecipitated from whole cell lysate (WCL), and from the soluble (Sol) and cytoskeletal (Cyt) fractions of Swiss 3T3 cells. As a control for non-specific precipitation equivalent lysates were subjected to the same procedure with the vinculin antibody omitted. The precipitate was boiled into protein loading buffer and resolved by SDS-PAGE, the products visualised by autoradiography. Representative of two experiments. Molecular weight marker (kDa) is shown on the left hand side of each panel.
Figure 5.20. Distribution of phosphorylated vinculin between the Triton X-100 soluble and cytoskeletal fractions. To analyse the distribution of phosphorylated vinculin between the Triton X-100 soluble and cytoskeletal fractions, $^{32}$P-orthophosphoric acid labelled cells were extracted with a 0.2\% or 1.0\% Triton solution for 2 minutes, as indicated. Vinculin was precipitated from whole cell lysate (WCL) and from the soluble (Sol) and cytoskeletal (Cyt) fractions of Swiss 3T3 cells. The precipitate was boiled into protein loading buffer and resolved by SDS-PAGE, the products visualised by autoradiography (A). The amount of vinculin immuneprecipitated, as determined by Coomassie stain is shown in (B). Representative of three similar experiments. Molecular weight markers (kDa) are shown on the left hand side of each panel.
Figure 5.21. (Top) Distribution of vinculin between the soluble and cytoskeletal fractions in serum-starved and LPA-stimulated Swiss 3T3 cells. To analyse the distribution of vinculin between the Triton soluble and cytoskeletal fractions, serum-starved (Starved) and 100 ng/ml LPA stimulated (LPA-stim) cells were extracted with a 0.2% Triton solution for 2 or 4 minutes, as indicated. Vinculin was precipitated from whole cell lysate (WCL) and the soluble (Sol) and cytoskeletal (Cyt) fractions. The precipitate was boiled into protein loading buffer, proteins resolved by SDS-PAGE and the vinculin detected by Western blotting with the precipitating antibody. Represents only one experiment. Molecular weight markers (kDa) are shown on the left hand side of the panel.

Figure 5.22. (Bottom) Distribution of vinculin between the soluble and cytoskeletal fractions of serum-starved Swiss 3T3 cells in suspension and cells spread on fibronectin. To analyse the distribution of vinculin between the Triton soluble and cytoskeletal fractions, serum-starved cells were trypsinised and maintained in suspension for 1 hour (Susp.) and spread on fibronectin coated dishes (50µg/ml) (Fibron.). Cells were then extracted with a 0.2% Triton solution for 2 or 4 minutes, as indicated. Vinculin was precipitated from whole cell lysate (WCL) and the soluble (Sol) and cytoskeletal (Cyt) fractions of Swiss 3T3 cells. The precipitate was boiled into protein loading buffer, proteins resolved by SDS-PAGE and the vinculin detected by Western blotting with the precipitating antibody. Represents only one experiment. Molecular weight markers (kDa) are shown on the left hand side of the panel.
Figure 5.21. Distribution of vinculin between the soluble and cytoskeletal fractions in serum-starved and LPA-stimulated Swiss 3T3 cells.

<table>
<thead>
<tr>
<th>WCL</th>
<th>2 min Triton</th>
<th>4 min Triton</th>
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<tr>
<td>Starved</td>
<td>LPA-stim</td>
<td>Starved</td>
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<tr>
<td>Sol</td>
<td>Cyt</td>
<td>Sol</td>
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Figure 5.22. Distribution of vinculin between the soluble and cytoskeletal fractions of serum-starved cells in suspension and cells spread on fibronectin.

<table>
<thead>
<tr>
<th>WCL</th>
<th>2 min Triton</th>
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<tr>
<td>Sol</td>
<td>Cyt</td>
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Figure 5.23. Tyrosine phosphorylated proteins including pp125Fak and paxillin are Triton extractable. Whole cell lysates or the Triton X-100 insoluble cytoskeletal fraction from cells extracted for 2 minutes with 0.2% Triton were boiled into protein loading buffer and equal amounts resolved by SDS-PAGE and transferred to nitrocellulose. The blots were subsequently probed with an anti-phosphotyrosine antibody (PT66) (A), then stripped and reprobed with the anti-human vinculin antibody (B). Lysates from a separate experiment were likewise probed for pp125Fak (C) and paxillin (D). Molecular weight markers (kDa) are represented on the left hand side of each panel.
Figure 5.24. The majority of tyrosine phosphorylated p125Fak and paxillin are Triton X-100 extractable. The distribution of tyrosine phosphorylated pp125Fak and paxillin between the soluble and cytoskeletal fraction of cultured (Cult), serum-starved (Starved) and LPA-stimulated (LPA-stim) Swiss 3T3 was determined by immunoprecipitation from lysates with an anti-phosphotyrosine antibody (PT66) conjugated to agarose beads, the immunoprecipitate boiled into protein loading buffer and then Western blotted with the monoclonal anti-pp125Fak and anti-paxillin antibodies. Cells were extracted with 0.2% Triton X-100 for 2 or 4 minutes as indicated. Represents only one experiment. WCL - whole cell lysate, Sol - represents the Triton soluble fraction, Cyt - Triton X-100 insoluble cytoskeleton. The broad intense band below paxillin is the precipitating IgG.
5.4.5 Phosphorylated vinculin does not become recruited to the cytoskeleton upon spreading of Swiss 3T3 cells on fibronectin

To determine whether vinculin became phosphorylated upon spreading on fibronectin, $^{32}$P-labelled serum-starved cells were trypsinised and kept in suspension in a polypropylene tube by occasional agitation or spread in tissue culture dishes coated with 50 µg/ml fibronectin for 60 minutes. The cells were then extracted with 0.2% Triton X-100 for 2 minutes. Even under these conditions the phosphorylated vinculin is mainly found in the cytosolic fraction (Fig. 5.25). Interestingly a strong band is apparent at 90 kDa. As the epitope recognised by the vinculin monoclonal antibody is reported to map to the head (Elena Moiseeva, personal communication), this band may represent the 90 kDa N-terminal head of vinculin which may become phosphorylated in vivo. From the in vivo labelling experiments, although vinculin is a phosphoprotein is unlikely that phosphorylation regulates the recruitment of the protein to focal adhesions.

5.4.6 Vinculin is a PKC substrate in vitro

As described in the introduction vinculin, has been shown to be regulated in vitro through an intramolecular interaction between the N-terminal globular head and the C-terminal tail (Johnson and Craig, 1994). To determine whether vinculin is indeed a substrate for PKC, it was assayed in vitro as a substrate for a preparation of mixed PKC isoforms. The sequences required for head-tail interaction and factors that regulated this interaction in vitro were also further defined. This work was performed in collaboration with Dr. John Weeke and the manuscript is presented in Appendix 1.

The ability of various PKC isoenzymes to phosphorylate chicken gizzard vinculin in vitro was investigated. Previous work in Dr. Peter Parker's laboratory (ICRF, London) demonstrated that the classical calcium-dependent isoforms of PKC, α, βI, βII and γ do not phosphorylate purified chick vinculin. The ability of the recombinant novel calcium-independent isoforms δ and ε to phosphorylate vinculin in vitro were subsequently assayed using full length vinculin, proteolytic fragments of vinculin produced by V8 cleavage, and fusion proteins spanning the length of vinculin as substrates. Only weak phosphorylation of the fragments corresponding to C-terminus could be observed with the δ isoform, but this phosphorylation was of very low stoichiometry (data not shown). The autophosphorylation of the enzyme used in these experiments was low, perhaps indicating that the activity of the enzyme was poor (data not shown). As none of the purified PKC isoforms tested appeared to phosphorylate vinculin, a crude preparation of PKC isoforms purified from bovine brain (Calbiochem, U.K.) was used to assay whether vinculin was indeed a substrate of PKC. This crude preparation consisted mainly of the calcium-dependent isoforms α, βI, βII and γ, with lower amounts of the other, less abundant, novel calcium-independent isoforms. Using purified chicken gizzard vinculin as a substrate, little incorporation of $^{32}$P-labelled phosphate into vinculin was observed (data not shown).
One possibility is that the PKC phosphorylation sites within vinculin are masked by the interaction between the head and the tail of the purified vinculin.

In the absence of a factor capable of disrupting a possible head-tail interaction, the vinculin was heated to 65°C for 30 minutes immediately before being assayed with the PKC mix (Fig. 5.26). This incubation would be expected to disrupt most protein-protein interactions. Incubation at 65°C did indeed result in the incorporation of more phosphate into vinculin though the stoichiometry was still very low (Fig. 5.26B), vinculin migrating just above the 116 kDa marker and the autophosphorylated PKC isoforms visible below the 97 kDa marker. The phosphorylated band in the final lane (Fig. 5.26B) is likely to be non-specific label which has not been washed away properly. As the same labelling does not occur in any other such PKC free sample in this and other similar experiments it is unlikely that this is due to a kinase co-purifying with vinculin. The failure to observe phosphorylation of full length vinculin is confusing as when the fusion proteins were used as a substrates, the C-terminal tail (residues 881-1066) became highly phosphorylated (Fig. 5.26C), though the other fusion (residues 1-398, 398-881) did not (see appendix 1). This implies that a PKC phosphorylation site lies in the tail of vinculin which is not accessible in the full length purified protein. Interestingly when the PKC assay was performed in the presence and absence of calcium, the ability of the PKC to phosphorylate vinculin was unchanged (Fig. 5.26C) implying that a calcium-insensitive isoform may be responsible for the phosphorylation of vinculin in vitro. Later work on the head-tail interaction revealed that acidic phospholipids inhibited head-tail binding. When intact purified chicken gizzard vinculin was preincubated with acidic phospholipids to relieve the head-tail interaction, and then assayed as a substrate for the PKC isoform mix then good PKC-dependent phosphorylation was observed (see appendix 1).

To determine whether the sites of PKC phosphorylation of intact vinculin heated to 65°C and the C-terminal fusion protein were the same, samples of each were analysed by cyanogen bromide (CNBr) digest and phosphoamino acid analysis. To facilitate this analysis the respective proteins were excised from the dried gels, the gel fragment rehydrated and then transferred to 0.2 μm PVDF by electroblotting. The digestion and hydrolysis reactions were then performed on the PVDF membrane. The full length vinculin and C-terminal vinculin fusion were subjected to CNBr digest overnight, the peptides eluted from the PVDF over several days and then resolved by electrophoresis, the labelled products detected by phosphorimaging. The only phosphorylated peptide obtained ran at the same position, below the 10 kDa marker, from both the purified intact vinculin and the C-terminal fusion protein (Fig. 5.27A).

To analyse the phosphoamino acid content of the two phosphorylated proteins the protein immobilised on PVDF membrane were hydrolysed by acid hydrolysis for 1 hour in 6M...
Figure 5.25. Distribution of phosphorylated vinculin in cells in suspension and spread on fibronectin. Serum-starved Swiss 3T3 cells were labelled with 100 μCi/ml $^{32}$P orthophosphoric acid in serum-free phosphate-free DMEM. They were then trypsinised, the trypsin inhibited with trypsin inhibitor, and cells were either maintained in suspension (Susp.) or spread on fibronectin (Fibron.) for 60 minutes. Vinculin was immuneprecipitated from lysates, the precipitate dissolved into protein loading buffer and resolved by SDS-PAGE. The phosphorylated products were visualised by autoradiography. Cells were extracted with 0.2% Triton for 2 minutes; Sol- represents the Triton X-100 soluble fraction, Cyt- represents the Triton X-100-insoluble cytoskeleton. Represents only one experiment. Molecular weight markers (kDa) are represented on the left hand side of the panel.
Figure 5.26. *In vitro* phosphorylation of vinculin by PKC. Purified chicken gizzard vinculin was incubated in a standard PKC assay to determine whether it is a substrate for PKC. Products of the reaction were resolved by SDS-PAGE and the phosphorylated products detected by phosphorimaging. A- Coomassie stained gel, B- Phosphorimage of phosphorylated products. Untreated vinculin or vinculin heated to 65°C was incubated with $^{32}$Pγ-ATP, in the presence and absence of PKC as indicated. Molecular weight markers are represented on the left of both panels. C- The C-terminal tail of vinculin (cleaved fusion protein) (residues 881-1066) was incubated with PKC in the presence (+) and absence (-) of calcium, the products resolved by SDS-PAGE and detected by phosphorimaging. The positions of PKC and the C-terminal vinculin fragment are indicated.
Figure 5.27. Analysis of the \textit{in vitro} PKC-mediated phosphorylation of vinculin.
Panel A- $^{32}$P-labelled phosphorylated chicken gizzard vinculin and phosphorylated C-terminal fusion protein cleaved from GST were electroblotted from rehydrated gels to 0.2 $\mu$m PVDF, CNBr digested and the peptides resolved by SDS-PAGE. The positions of the phosphorylated peptides were visualised by phosphorimaging. Panel B-Phosphorylated purified chick gizzard vinculin and phosphorylated C-terminal fusion were likewise transferred to PVDF membrane and then hydrolysed in 6 M HCL for 1 hour at 110°C and the amino acids resolved by TLC. The positions of the amino-acid standards are indicated. The phosphorylated amino acids were visualised by phosphorimaging.
A. Purified full length vinculin protein

B. Purified full length vinculin protein compared to C-terminal fusion protein:
- Free Phosphate
- Phosphoserine
- Phosphothreonine
- Phosphotyrosine
HCl at 110°C. The phosphoamino acids were then resolved by TLC and the position of the labelled amino acid detected by phosphorimaging (Fig. 5.27B). The only labelled phosphoamino acid detected in both digests was phosphoserine, though again less labelled product was recovered from the labelled intact vinculin, a reflection of the fact that less phosphate was incorporated into the starting product. The results from the in vitro analysis of the phosphorylation of vinculin implies that PKC phosphorylates the protein on serine residue(s) within the C-terminal tail. Further work has shown that the phosphorylation site is hidden in the native full length protein, and is revealed upon incubation with acidic phospholipids prior to assay.

5.5 Discussion
Role of PKC
Evidence implicating a role for PKC in the regulation of cell adhesion has come mainly from pharmacological approaches. In Swiss 3T3 cells, it appears that activation of PKC with TPA inhibits the LPA-mediated formation of focal adhesions and actin stress fibres in serum-starved Swiss 3T3 cells. TPA induced the formation of membrane ruffles and small clusters of phosphotyrosine-containing proteins at the plasma membrane. However TPA also stimulates the tyrosine phosphorylation of a similar spectrum of cellular proteins to that induced during the LPA response, one of which was identified as pp125Fak. The effect of TPA on the tyrosine phosphorylation of cellular proteins was not enhanced by treatment of cells with LPA. TPA treatment also induced the loss of actin stress fibres and vinculin-containing adhesions from normal cultured cells. Conversely inhibiting PKC with the PKC-specific inhibitor calphostin C induced rounding of serum-starved cells with a concomitant formation of disorganised actin filaments. Moreover LPA did not prevent the calphostin C-mediated cell rounding. For clarity the effects of activating and inhibiting PKC in Swiss 3T3 cells are summarised in Fig. 5.28.

The ability of TPA to induce disruption of the actin cytoskeleton has been observed in several cell types. In BSC-1 cells TPA treatment results in a loss of α-actinin, talin and vinculin from focal adhesions, as well as disruption of the actin cytoskeleton (Meigs and Wang, 1986; Turner et al., 1989) TPA has also been reported to promote fibroblast and CHO cell spreading on ECM components (Woods and Couchman, 1992; Vuori and Ruoslahti, 1993). It is possible that the loss of actin stress fibres and discernable focal adhesions seen upon treatment of cells with TPA (Meigs and Wang, 1986; Turner et al., 1989) is indicative of a cell spreading response, and enables the cell to become more closely apposed to the matrix. This argument can be applied to the responses observed in Swiss 3T3 cells. Actin stress fibres and focal adhesions make cells contracted and bulky, adherent to the matrix at only a few strong points of close contact (focal adhesions). Promoting cell spreading would be predicted to induce a phenotype indistinguishable from that of serum-starved cells. A well spread cell, containing no such structures, is
LPA-stimulated cell
Actin stress fibres and focal adhesions

Activation of adhesive machinery. Cell contraction coupled with increased focal adhesion formation

Serum-starved cell
No actin stress fibres/focal adhesions

Calphostin C TPA
Formation of small adhesive clusters

Inhibition of PKC
Cell contracted, or rounded. Short actin filaments and small focal adhesions formed

PKC activation
Membrane ruffles. Spreading promoted?

Fig. 5.28. Summary of the effects of inhibiting and inactivating PKC in serum-starved Swiss 3T3 cells. Actin is shown in green and focal adhesions in red.
presumably adherent at more point contacts perhaps not visible by immunofluorescent techniques. Although treating serum-starved Swiss 3T3 with TPA does not induce a phenotype change other than membrane ruffling, this may be misleading. Indeed when normal cultured Swiss 3T3 cells are treated with TPA it induces a loss of focal adhesions and stress fibres, with the cells assuming a serum-starved phenotype. The cells lose the classical pointed fibroblastic-shape and become more rounded.

The phosphotyrosine clusters formed upon treating serum-starved cells with TPA may represent small point contacts generated as cell spreading is promoted, possibly analogous to focal structures formed upon microinjection of cells with rac (Nobes and Hall, 1995b). The ability of TPA to induce the tyrosine phosphorylation of cellular proteins associated with the formation of adhesive structures may also be indicative of the formation of new points of contact with the ECM, perhaps through the activation of integrins which engage the ECM as spreading is promoted. The TPA-mediated tyrosine phosphorylation of pp125Fak and paxillin has been described independently in confluent quiescent but not serum-starved Swiss 3T3 cells (Sinnett-Smith et al., 1993), and TPA also enhances the tyrosine phosphorylation of pp125Fak in CHO cells spreading on fibronectin (Vuori and Ruoslahti, 1993). In support of this hypothesis TPA has previously been shown to induce the inside-out signal required for activation of the LFA-1 integrin complex in lymphocytes (Tominaga et al., 1993). One important observation is that TPA treatment of Swiss 3T3 cells does not inhibit the formation of actin stress fibres induced by microinjection of rhoA. This implies that TPA overrides the LPA-induced signal at some point upstream of rho (Ridley and Hall, 1994).

The PKC inhibitor bisindolylmaleimide has previously been shown to inhibit the TPA-mediated tyrosine phosphorylation of pp125Fak and paxillin in quiescent Swiss 3T3 cells (Sinnett-Smith et al., 1993; Seufferlein and Rozengurt, 1994). When the effect of this inhibitor on the cytoskeleton was assessed by immunofluorescence, aggregates could be seen around the nuclear region, possibly indicating that it was insoluble or localised to a specific subcellular location. Use of the alternative PKC inhibitor calphostin C proved more informative. The ability of calphostin C to round up the serum-starved Swiss 3T3 cells implies that a kinase sensitive to calphostin C is required for maintaining a spread phenotype. At the concentrations used and under the conditions of the experiments, calphostin C preferentially inhibits PKC isoforms (Kobayashi et al., 1989; Bruns et al., 1991). This observation correlates with previous evidence that activation of PKC in spreading CHO cells is required for integrins to engage ligand (Vuori and Ruoslahti, 1993). In Chapter 4 the serum-starved Swiss 3T3 were shown to adhere in an integrin-dependent manner, which implies that PKC activity is required to maintain the attachment of serum-starved cells possibly mediating an inside-out signal, controlling the affinity of the integrins for the ECM.
The ability of calphostin C to stimulate the formation of short actin filaments is confusing. Although these filaments stain strongly with phalloidin, they are not associated with "mature" focal adhesions, but small adhesive clusters as determined by vinculin and phosphotyrosine staining. Data presented here collectively suggests that PKC may act, not by directly regulating proteins such as vinculin and α-actinin listed above, but by controlling some other aspect of the adhesive machinery, perhaps negatively regulating the contractile apparatus of the cell. The ability of PKC to promote cell spreading, presumably increasing the number of cell-matrix interactions, could stimulate tyrosine phosphorylation of cellular proteins through increased integrin activation. Indeed, inhibiting PKC in serum-starved Swiss 3T3 cells with calphostin C induces not only the appearance of short actin filaments, but also the contraction of the cell body and the formation of retraction fibres. If PKC plays a role in negatively regulating the contractile apparatus of the cell, then relieving this control when the cell is in a weakened adhesive state may well be enough to induce the cell contraction and subsequent rounding observed. This contraction could perhaps be increased upon the addition of LPA, rather than inhibited because the interaction of integrins with the ECM has been inhibited. The short actin filaments may arise through condensation of fine actin filaments as the cells contract. A network of fine actin filaments may be present in the serum-starved cells, but cannot be detected by phalloidin staining under the fluorescence microscope. Moreover the small focal adhesions formed in calphostin C treated Swiss 3T3 cells may represent the immature adhesive links through which the serum-starved cells are interacting with the ECM, and contraction of the cell may cluster these complexes into structures visible with the fluorescence microscope.

**Myosin II, a possible target of PKC?**

Possible adhesion-related targets of PKC may include components of the actomyosin complex, in particular myosin II a double headed long rod-like molecule capable of polymerising into bipolar filaments. It can bind to actin filaments and produce an ATP-dependent motion, acting as a molecular motor. It is abundant in the cell cortex, and as bipolar filaments is capable of pulling two actin filaments past each other producing a contraction similar to that observed in muscle (Lauffenburger and Horwitz, 1996). Myosin II consists of two chains, the myosin heavy chain (MHC) and the 20 kDa myosin light chain (LC20). In the dephosphorylated state LC20 inhibits myosin II, downregulating the ATPase activity of the MHC. However phosphorylation by myosin light chain kinase (MLCK) relieves this inhibition and activates the contractile properties of myosin II (reviewed in Tan et al., 1992). In LPA- and serum-stimulated Swiss 3T3 fibroblasts myosin II is distributed along the length of actin stress fibres (Giuliano et al., 1992; Ridley and Hall, 1994), and in thrombin-stimulated human umbilical vein endothelial (HUVE) cells is associated with the reorganisation of actin into filaments (Goecckeler and
In migrating REF52 cells nascent clusters of myosin II form at sites of cell retraction, and appear to organise disordered actin filaments into stress fibres by forming a myosin filaments which appear to co-align the F-actin into bundles by pulling the individual filaments (Verkhovsky et al., 1995). Myosin II therefore has potential to act as part of a contractile complex to generate cytoplasmic tension. Indeed it is distributed in a punctate manner along actin filaments, considered to be contractile, at sites which alternate with the distribution of the bundling protein α-actinin.

A correlation between the MLCK-mediated phosphorylation of LC20 and the formation of actin stress fibres has been described in a number of cell types including HUVE (Goeckeler and Wysolmerski, 1995), CEF (Kolodney and Elson, 1995), Swiss 3T3 (Giuliano et al., 1992) and thyroid cells (Deery and Heath, 1993). Moreover a low basal phosphorylation of LC20 is required to maintain stress fibres integrity in Swiss 3T3 cells (Lamb et al., 1988; Fernandez et al., 1990). Upon serum-deprivation (relaxation) of Swiss 3T3 cells the phosphorylation of LC20 decreases, but rapidly increases again upon the readdition of serum associated with contraction of the cell and the reformation of actin stress fibres (Giuliano et al., 1992). Interestingly cell contraction and shortening can be mimicked by addition of the phosphatase inhibitor okadaic acid, and serum-induced contraction inhibited by the broad range kinase inhibitor staurosporine (Giuliano et al., 1992). In thyroid cells, TPA treatment induces the dissolution of organised actin stress fibres which is associated with the dephosphorylation of LC20 at residues corresponding to sites of MLCK phosphorylation (threonine 18 and serine 19). TPA did not induce this dephosphorylation of LC20 in the presence of the phosphatase inhibitor calyculin A implying that TPA excerts an effect indirectly by activating a serine/threonine phosphatase that dephosphorylated LC20, so inactivating myosin II (Deery and Heath, 1993). It is tempting to speculate that similar events occur when starved and cultured fibroblasts are treated with TPA or calphostin C. TPA treatment could act on two levels. PKC activation promotes spreading through activation of integrins by generating an inside-out signal, which in turn stimulates protein tyrosine phosphorylation (Vuori and Rouvalahti, 1993). The increased integrin engagement may occur because PKC also activates a phosphatase that relaxes the cell's contractile apparatus by dephosphorylating and inhibiting myosin II. This argument could be used to explain why TPA induces dissolution of the actin architecture and focal adhesion integrity in cultured cells. PKC activation relaxes the actin filament organisation by inhibiting myosin II, which plays a role in gathering unbundled filaments. Moreover it promotes spreading by increasing the adhesive potential of the cell through inside-out activation of integrins.

The converse would occur when cells are treated with calphostin C. Inhibition of PKC results in a decrease of the phosphatase activity responsible for regulating myosin II by dephosphorylating LC20. Basally active MLCK is then able to activate myosin II and
induce cell contraction. However in the absence of established focal adhesions and organised actin cytoskeleton the cell is too weak to resist the contraction and cell rounding is induced as the cell pulls away from the matrix. This effect could be enhanced as inhibiting PKC may also decrease the affinity of the unclustered integrins for the ECM. Interestingly, it has been speculated that in a migrating cell, myosin II induced contraction is responsible for breaking the weak adhesive links at the rear of the cell (Jay et al., 1995). This is a situation which is perhaps analogous to that in serum-starved cells treated with calphostin C, where adhesion appears to be mediated by numerous weak adhesive links.

In serum-starved Swiss 3T3 cells TPA inhibits the LPA-mediated formation of focal adhesions and actin stress fibres, but not the stress fibres stimulated by the microinjection of constitutively active rho (Ridley and Hall, 1994). This implies that to inhibit stress fibre formation TPA inhibits or overrides a signalling event upstream of rho. However, it is possible that rho and PKC are competing for the same target. In permeabilised vascular smooth muscle cells, addition of GTPyS induces the phosphorylation of LC20, in a rho-dependent manner, through inhibition of a phosphatase (Noda et al., 1995). Microinjection of constitutively active rho may be sufficient to overcome the effect of TPA, and competitively inhibit the phosphatase so activating myosin II. Alternatively it is possible that TPA also inhibits rho, perhaps by inhibiting nucleotide exchange, and that microinjection of constitutively active rho loaded with GTP overcomes this constraint. These two models are summarised in Fig. 5.29.

If this model were applied to spreading cells then a hierarchy of signalling events could be envisaged. When cells spread on fibronectin there is an initial burst in PKC activity, thought to occur before integrins engage the ECM (Vuori and Rouslahti, 1993). This burst of PKC activity could serve a number of functions. Firstly it activates integrins through an inside-out signal and secondly it inhibits the contraction of the cell while it has weak adhesive links during the first few minutes of spreading. Once the cell has established points of adhesion and the PKC activity falls, rho-mediated contraction and stress fibre formation can occur without the cell pulling off the matrix. How PKC is activated in adhesion related responses is unclear. One possible pathway facilitating PKC activation via integrins has however been identified. HeLa cell adhesion to collagen activates an arachadonic acid-mediated signalling pathway which leads to the activation of PKC and is required for cell spreading on fibronectin (Liu et al., 1991; Chun and Jacobson, 1992; Chun and Jacobson, 1993). Using specific inhibitors of PLA₂, lipoxygenase and PKC the same pathway has been shown to be stimulated upon the clustering of β1-integrin in HeLa cells with antibodies (Auer and Jacobson, 1995). The activation of PKC is thought to be mediated by lipoxygenase-generated second messengers that activate PLC, thereby activating PKC via the generation of DAG and the release of intracellular calcium. Interestingly, cells treated with calphostin C were able to attach but not spread on substrate
1. Activation of PKC in serum-starved Swiss 3T3 cells

- TPA → PKC
- Inside-out activation of integrins
- Activation of phosphatase → Stimulation of rhoA
- Engagement of the ECM
- Dephosphorylation of LC20
- Inhibition of myosin II
- Formation of actin stress fibres and focal adhesions
- Tyrosine phosphorylation of pp125Fak and paxillin etc.
- Increased cell-spreading
- Activation of pp125Fak and paxillin tyrosine phosphorylation as a result of increased integrin-activation

2. Inhibition of PKC in serum-starved Swiss 3T3 cells

- Calphostin C → PKC
- Decrease in integrin engagement of ECM
- Decreased phosphatase activity
- Increase in LC20 phosphorylation
- Activation of myosin II
- Cell contraction results in cell rounding because of weak adhesive links
- Decreased phosphatase activity

Figure 5.29. Schematic representation of the possible signalling events resulting from activation and inhibition of PKC in serum-starved Swiss 3T3 cells.
components, perhaps indicating that PKC operates in parallel with tyrosine kinases to generate a spread cell containing focal adhesions and actin stress fibres (Vuori and Ruoslahti, 1993). Recently the PKC-mediated aspects of cell adhesion have been greatly complicated with the discovery that the novel PKC-related Protein kinase N family is activated by rhoA, as well as by TPA (Amano et al., 1996; Watanabe et al., 1996). Whether this kinase exerts a rho regulated effect on the cytoskeleton remains to be established.

A major problem associated with studying PKCs using broad range pharmacological activators such as TPA, or inhibitors such as calphostin C, is that more than one isoform is affected. It is possible that different PKC isoforms regulate different aspects of the signalling pathways involved in cell adhesion. Although pharmacological approaches can provide a valuable indication of possible cellular roles of members of the PKC family, they do not allow the function of specific PKC isoforms to be dissected. As a result the evidence that PKC isoforms are involved in adhesion regulation could be regarded as purely circumstantial, as no individual PKC isoform has been formally shown to be required. The specific role of PKC in adhesion can only be properly addressed by modulating the expression of each individual PKC isoform, by overexpression, antisense downregulation/gene knockout, or by microinjection of isoform specific inhibitors into cells. It is possible that isoform specific inhibition could be achieved by expressing or microinjecting the regulatory domains of the individual isoforms. Only four PKC isoforms are expressed in Swiss 3T3 fibroblasts (Olivier and Parker, 1994), which simplifies the task of identifying whether PKC isoforms are directly involved in regulating the interaction of cells with the ECM. Such an approach could be coupled with a careful assay of changes in activity of specific PKC isoforms during the adhesive response. In conclusion adhesion depends on the integration of innumerable signals which have to activate the cellular machinery in carefully orchestrated hierarchies to obtain the correct response.

**Localisation of PKC to focal adhesions**

If PKC plays a role in adhesion regulation, then localisation of a PKC isoform to sites of adhesion provides strong circumstantial evidence for this. Indeed PKCα localises to focal adhesions in REF52 cells (Jaken et al., 1989). Although this has not been confirmed in any other fibroblast cell line, PKCα has now been localised to adhesive structures in macrophages (Allen and Adarem, 1995). As PKCα did not localise to focal adhesions in Swiss 3T3 cells, the possibility that other PKC isoforms localises to adhesions was investigated. A commercial antibody raised against PKCδ marketed by Transduction Laboratories stained fibroblasts cells lines with a pattern reminiscent of focal adhesions. Staining Swiss 3T3 cells with this antibody confirmed that it did indeed recognise a protein localised to the ends of actin stress fibres extending from focal adhesions. The focal adhesions staining was lost in serum-starved cells with the antibody staining the nuclear
region, but restored upon stimulation with LPA and FCS. Interestingly the localisation of the reactive protein to focal adhesions was only seen after 5 minutes of stimulation with LPA. This is unusual as all the other focal adhesion proteins studied can be detected in focal complexes after only 2 minutes. It appears that the protein recognised by the PKCô antibody localises to adhesive structures only when actin stress fibres have become discernable. Interestingly it also stains granular structures within the cytoplasm.

More recently, the possibility has arisen that the PKCô antibody recognises a protein of 80 kDa that is not PKCô, and because of these doubts has been removed from sale. Evidence for this is that expression of the 80 kDa band recognised by the antibody does not downregulate upon prolonged exposure of cells to TPA, a feature common to PKC isoforms (Peter Parker, personal communication). Therefore to determine whether PKCô does localise to focal adhesions, the distribution of the protein was investigated by overexpressing rat PKCô in monkey Cos cells. As the Cos cells did not contain many actin stress fibres a specific subcellular localisation of the expressed protein by transient expression was not identified. Overexpression of the correct PKC isoform could induce disruption of the actin cytoskeleton, however this is unlikely in this case as the majority of the untransfected Cos cells also possessed few or no actin stress fibres. The observation that the monoclonal anti-PKCô antibody from Transduction Laboratories also recognises an 85 kDa band in Cos cells, not recognised by the polyclonal anti-PKCô antibody, may support the idea that the antibody is not as specific as previously thought.

Given the doubt associated with identifying the localisation of PKCô by direct staining with the PKCô antibodies, the simplest way to confirm its localisation seemed to be by expressing epitope tagged PKCô in mouse fibroblasts. The rat PKCô cDNA was therefore fused to a myc epitope tag and expressed in Balb/c 3T3 cells, either constitutively using the pCDNA-3 mammalian expression vector, or using the inducible pMAM-neo BLUE mammalian expression vector. Unfortunately none of the clones obtained from transfection of either of these constructs gave high enough expression to be detected by Western blotting. As a result no subcellular location could be determined by immunofluorescence. The failure to detect expression of the tagged PKC could be due to a number of reasons. The protein could be transcriptionally regulated such that initial overexpression of protein results in downregulation over a period of time. Significantly very few stable cell lines expressing active PKCô have been successfully isolated in any other laboratories (Peter Parker, personal communication), so in retrospect this result is not entirely surprising. It is possible that the construct may have integrated into a transcriptionally quiet part of the genome, though as the cells are G418 resistant this is unlikely. Alternatively there may be a problem with both constructs that prevents expression of the proteins, although the ends of both were sequenced to ensure that the correct reading frames were maintained throughout the construction process. The epitope
tagged PKCδ could be transiently expressed from the pCDNA-3 construct which generally result in higher levels of exogenous protein production, and may allow identification of the subcellular localisation of PKCδ. Confirmation of which PKC isoforms localise to focal adhesions in a variety of cell types is also required to further determine if PKC plays a role in assembling the focal adhesions and if so which isoforms may be involved. In conclusion it has not been possible to confirm whether or not PKCδ localises to focal adhesions.

The distribution of PKCα has been further studied in REF52 cells. Although PKCα localises to focal adhesions and is tightly associated with the cytoskeleton in normal REF52 cells, upon transformation it is no longer localised to focal adhesions, but becomes associated with the Triton X-100 insoluble fraction (Hyatt et al., 1990). In both normal and transformed REF52 cells the protein also appeared to localise to the nucleus though this was not discussed by the authors. In both serum-starved and LPA-stimulated Swiss 3T3 cells, the PKCα antibody used only showed a cytosolic distribution with some localisation to the nucleus. Although this antibody recognised PKCα by Western analysis the failure to reveal a specific cellular location for PKCα implies that it may not localise to focal adhesions in Swiss 3T3 cells. It is possible that not enough PKCα is expressed for the localisation of the protein to be determined. Alternatively the method of fixation may not allow the localisation to be revealed.

The roles of both PKCα and PKCδ in cellular transformation have been investigated. Expression of PKCδ, but not PKCα and ε, was elevated in clonally selected SV40 transformed REF52 cells that possessed the ability to grow in soft agar, suggesting that PKCδ facilitated anchorage dependent growth. Expression of the regulatory domain PKCδ inhibited growth in soft agar while expression of the PKCα regulatory domain increased growth in soft agar (Liao et al., 1994b). Overproduction of endogenous PKC isoforms in cells partially transformed with adenovirus E1A, and cells transformed with H-ras results in a more complete transformed phenotype (Hsiao et al., 1989; Su et al., 1991; Su et al., 1992). Moreover, overexpression of PKCδ and PKCε in NIH 3T3 and CHO cells indicated that PKCδ was associated with growth arrest, while PKCε was associated with anchorage independent growth (Watanabe et al., 1992; Mischak et al., 1993). These results suggest that both the α and δ isoforms of PKC have effects on cell growth, though whether this is through regulation of the cytoskeleton or adhesive machinery is unclear.

PKC isoforms have further been shown to interact with a number of intracellular proteins. One group is the RACKs, which are small proteins (30 to 33 kDa) which interact with phosphatidyl serine and with PKC at a site distinct from the substrate binding site. These proteins are thought to be responsible for the translocation of activated PKC to the membrane (Mochley-Rosen et al., 1991a; Mochley-Rosen et al., 1991b; Ron et al., 1994).
The second group are larger proteins of 110 and 115 kDa which also bind to protein kinase C in the presence of phosphatidyl serine, but are enriched in cytoskeletal fractions (Wolf and Sahyoun, 1986). Indeed PKCα has been shown to interact with annexins, vinculin and talin by chemical cross-linking in vitro (Hyatt et al., 1994), an interaction that appears to be determined in part by the pseudosubstrate domain (Liao et al., 1994a). The demonstration that PKC can interact with certain cytoskeletal proteins offers an explanation as to why PKCα may localise to focal adhesions. This does not however rule out the possibility that other PKC isoforms may localise or be involved in adhesion regulation. The major problem involved in studying PKC is the number of different isotypes of the enzyme, and also the emergence of other enzymes related to PKC.

Phosphorylation of vinculin and recruitment to the cytoskeleton

Despite a lack formal proof, phosphorylation of vinculin has long been thought of as a mechanism that modulates the recruitment of the protein to sites of adhesion. In Swiss 3T3 cells, although vinculin is clearly a phosphoprotein, the stoichiometry of this phosphorylation is low and occurs mainly on serines (Sefton and Hunter, 1981). Previous studies have shown that vinculin is a phosphoprotein in vivo, with the phosphorylation increased upon treatment of cells with TPA. However in Swiss 3T3 cells, no such increase was observed, an observation also reported for vinculin in monkey BSC-1 cells (Turner et al., 1989). Moreover data published by Geiger (1982) suggested that the phosphorylated vinculin was associated predominantly with the Triton X-100 insoluble cytoskeleton, rather than the soluble fraction. However under the conditions used in this study the phosphorylated vinculin appeared associated with the Triton X-100 soluble fraction. This observation was confirmed using Balb/c 3T3 cells (data not shown). Under the conditions of fractionation between 20 and 30% of the total vinculin appeared associated with the cytoskeleton, while almost all the phosphorylated vinculin was solubilized by the extraction procedure. The proportion of cellular vinculin associated with the insoluble fraction is higher in Swiss 3T3 than that originally described by Geiger (1982) in chick embryo fibroblasts, but this could be due to the variation between cell lines and fractionation techniques.

Significantly, no increase or redistribution of the phosphorylated vinculin could be detected when serum-starved cells were either stimulated with LPA or when spread upon fibronectin. Both experiments were performed in serum-free media. It is possible that incorporation of phosphate into cytoskeletal-associated vinculin could occur when cells are spread in the presence of serum. However the failure to detect high stoichiometric phosphorylation of vinculin, or changes in vinculin phosphorylation under any experimental conditions implies that phosphorylation is not significant in recruitment to the cytoskeleton. It is also possible that the vinculin associated with the cytoskeleton in fibroblasts does not turn over quickly, therefore new vinculin may not be recruited to the
cytoskeleton under the conditions of these experiments. As a result labelled phosphate may not be incorporated into protein associated with the Triton X-100 insoluble fraction, even though phosphorylation may be required for the recruitment of vinculin to this subcellular fraction. Despite this the data suggests that in Swiss 3T3 cells, phosphorylation of vinculin is low and is not associated with recruitment of the protein to the Triton X-100 insoluble cytoskeleton.

Interestingly analysis of the changes in vinculin distribution reveals that under the fractionation conditions employed, vinculin remains associated with the Triton X-100 insoluble cytoskeleton even in serum-starved cells or cells taken into suspension by trypsinisation. This implies that even under conditions where the cytoskeleton and focal adhesions would be expected to be disrupted a proportion of the vinculin remains associated with an organised cytoskeleton perhaps allowing for rapid incorporation into adhesive structures. The majority of work examining the redistribution of proteins between the cytosolic and cytoskeletal fractions has been performed in platelets, which allows the mobilisation of cytoskeletal proteins during the adhesive response to be studied. Stimulation of platelets with fibrinogen or thrombin induces a redistribution of proteins including integrins, vinculin, talin and pp125Fak from a cytosolic to a cytoskeletal location, as determined by Triton X-100 fractionation of cells (Beckerle et al., 1989; Vostal and Shulman, 1993; Bertagnolli et al., 1993a; Bertagnolli and Beckerle, 1993b). Both vinculin and talin have been reported to be tyrosine and serine/threonine phosphorylated upon activation of platelets (Vostal and Shulman, 1993), but are not tyrosine phosphorylated in mouse fibroblasts (Barry and Critchley, 1994; Bockholt and Burridge, 1993). Vinculin at least, also appears poorly phosphorylated on serine/threonine residues (this study; Turner et al., 1989). Platelets are essentially suspension cells, which circulate, adhesively inert in the blood, only mounting an adhesive response when activated at sites of wounding. Fibroblasts on the other hand are adhesive cells requiring an interaction with the matrix for survival, and so must always maintain a link between the actin cytoskeleton and the ECM. It is possible that the Swiss 3T3 cells never disassemble the cytoskeleton, but rather maintain organised subcellular pools of proteins complexed with short actin filaments, primed, ready to mount an adhesive response.

Under the same fractionation conditions it appears that neither the tyrosine phosphorylated forms of pp125Fak or paxillin become stably associated with the Triton X-100 insoluble fraction upon LPA-stimulation. More detailed analysis by immuneprecipitation of tyrosine phosphorylated proteins from the various fractions of serum-starved cells and LPA-stimulated cells reveals that the majority of the tyrosine phosphorylated pp125Fak and paxillin is soluble, though a proportion does appear associated with the Triton X-100 insoluble cytoskeleton. This suggests that the tyrosine phosphorylated forms of pp125Fak and paxillin may interact with the structural components of the focal adhesions such as β1-
integrin (Schaller et al., 1995) and talin (Chen et al., 1995) but are not integrally structural components of the focal adhesions and actin cytoskeleton.

It must be stressed that Triton X-100 extraction has only been used as a crude method of determining the relative distribution of proteins of interest between the soluble and insoluble compartments. The data obtained strongly suggests that in Swiss 3T3 cells phosphorylation of vinculin is not associated with the recruitment of the protein to the cytoskeleton. Moreover no substantial redistribution of vinculin to the soluble fraction was observed upon disruption of cell adhesion. It would be informative to analyse the changes in the other cytoskeletal proteins such as talin, α-actinin, tensin, paxillin and pp125Fak in detail using this fractionation technique. More quantitative analysis of the distribution of proteins between the cytosolic and cytoskeletal compartments under conditions of adhesion formation would yield clues as to how fibroblasts regulate cell adhesion. It is likely that the hierarchy of events in adherent cell types differs greatly from events in suspension cell types such as platelets that only become adherent under extreme conditions.

**Activation of vinculin in vitro**

Central to understanding vinculin activation was the discovery that the N-terminal globular head of the protein makes an intramolecular interaction with the C-terminal tail (Johnson and Craig, 1994; Johnson and Craig, 1995a). Initial attempts to repeat the data of Werth and Pastan (1983) i.e. to phosphorylate purified chick vinculin with PKC in vitro were unsuccessful. This was probably because possible sites of phosphorylation are masked in the full length protein because of an association between the head and tail of vinculin. Heating vinculin before assaying as a substrate for PKC isoforms led to increased incorporation of phosphate into the protein, perhaps through perturbation of the head-tail interaction in a proportion of the vinculin molecules. Limited proteolysis and phosphoamino acid analysis revealed that the full length vinculin and C-terminal region of vinculin are phosphorylated on serine residues within the same peptide fragment. Interestingly the ability of the mix of PKC isoforms to phosphorylate the C-terminal tail of vinculin in the absence of calcium may indicate that the PKC isoform(s) active against vinculin is a calcium-insensitive novel isoform.

Unfortunately, as the level of phosphorylated vinculin observed in vivo was very low it was not possible to analyse the sites or nature of the phosphorylation. Inspection of PKC consensus sequences together with the information obtained from CNBr cleavage reveals that the phosphorylated fragment probably corresponds to residues 934-1006. Within this fragment are two serines (941 and 999) within the context for phosphorylation by PKC. This needs to be formally confirmed by peptide sequencing of the fragment. Analysis of the C-terminal region of vinculin reveals consensus phosphorylation sites for not only PKC, but also cAMP-dependent kinase, cGMP-dependent kinase, AMP-activated protein kinase and calcium-calmodulin kinase II.
kinase, casein kinase II and proline-dependent protein kinases. Further work has revealed that the C-terminal tail is not phosphorylated by either casein kinase II or the AMP-activated kinase. Although the data presented above establishes that vinculin is a putative substrate for PKC in vitro the inability to incorporate phosphate into the purified vinculin, further supports the conclusion that phosphorylation is not responsible for recruitment of vinculin to focal adhesions.

The interaction between the head and tail of vinculin also masks the binding sites for both F-actin and α-actinin (Johnson and Craig, 1994; Kroemker et al., 1994; Johnson and Craig, 1995a). The failure to phosphorylate full length vinculin with PKC in vitro is therefore probably because the vinculin purified from chicken gizzard used for these assays is in the inactive state. The head-tail interaction is mediated by residues (1029-1036) in the tail, but the region in the head is less well defined lying within residues 1-258, with residues required for talin binding (167-207) (Gilmore et al., 1992; Gilmore et al., 1993), also required. The PKC phosphorylation site(s) may also be masked by this interaction.

Further in vitro work on the intramolecular regulation of vinculin (see appendix 1) revealed that this interaction is relieved by binding of acidic, but not neutral phospholipids. Preincubation of purified chicken gizzard vinculin with acidic phospholipids not only reveals the F-actin binding site, but also phosphorylation of vinculin by the PKC isoform mix. The C-terminal tail of vinculin has recently been shown to contain a cryptic binding site for acidic phospholipids (916-970) (Johnson and Craig, 1995b). Computer modelling predicts acidic phospholipid binding sites within residues 935-978 and 1020-1040 (Temple et al., 1995). Experimental proof for the second phospholipid binding site is yet to be published. Vinculin also interacts with micelles consisting of acidic phospholipids but not to neutral phospholipids, such as phosphotidyl choline (Niggli et al., 1990; Niggli and Gimona, 1993), and to vesicles containing PIP2 with a higher affinity than those containing other phospholipids. By labelling with photoactivatable probes, an interaction of vinculin with acidic phospholipids has also been observed in cultured fibroblasts, suggesting that this may also be a relevant interaction in vivo, as well as in vitro (Goldmann et al., 1992). Moreover both vinculin and α-actinin have been co-immunoprecipitated complexed to acidic phospholipids (Fukami et al., 1994). The N-terminal head of vinculin is acidic (pl 5.4) while the tail is basic (pl 9.7). The interaction of acidic phospholipids with the C-terminal tail could therefore change the overall charge of the region and disrupt the interaction with the head. Vinculin may be activated in vivo by interacting with PIP2-rich regions in the lipid bilayer, or by transfer of PIP2 to the protein. Interestingly growth factors such as PDGF decrease the cellular concentration of PIP2 (Fukami et al., 1994), and also transiently disrupt focal adhesions (Herman and Pledger, 1985). The subsequent phosphorylation of the vinculin tail by PKC, or binding of other proteins such as talin, actin and possibly paxillin could serve to lock the protein in
the open active form. However the distribution of the phosphorylated vinculin in the soluble fraction suggests that phosphorylation may play a role in removing vinculin from cytoskeletal structures.

The possibility that acidic phospholipids may play a role in regulating cell adhesion is an attractive one. Rho proteins which regulate the formation of cytoskeletal structures stimulated by both integrins and factors such as LPA, also regulate the activity of phosphatidylinositol 4-phosphate 5-kinase in mammalian cells (Chong et al., 1994), further supporting the hypothesis that acidic phospholipids such as PIP2 could regulate vinculin. The same may be true of a number of other cytoskeletal proteins. The actin cross-linking activity of α-actinin has been shown to be enhanced by PIP2 (Fukami et al., 1992). Computer analysis predicts that mouse talin contains putative phospholipid binding sites within the 47 kDa N-terminus, at residues 21-39; 287-342 and 385-406, with a general lipid-binding domain between residues 385-401 (Tempel et al., 1995). Moreover in vitro analysis of talin suggests that it also interacts with phospholipid membranes, supported by observation made upon the activation of platelets, where talin interacts with the membrane in the absence of functional integrin (Bertagnolli et al., 1993a). Numerous in vitro studies have demonstrated an interaction between talin and acidic phospholipids (Goldmann et al., 1992; Kaufmann et al., 1992; Dietrich et al., 1993). Interestingly electron microscopy has raised the suggestion that like vinculin, talin also undergoes a degree of intramolecular interaction (Isenberg and Goldman, 1992), while in its biologically active state is a dumbbell shaped homodimer (Goldmann et al., 1994).

In summary data presented in this chapter strongly suggests that PKC plays a fundamental role in regulating the interaction of Swiss 3T3 cells with the ECM. It further demonstrates activation of PKC with TPA induces tyrosine phosphorylation of cellular proteins including pp125Fak, and that PKC function is required to maintain the spread phenotype of serum-starved Swiss 3T3 cells. The manner in which PKC regulates cell adhesion is unclear, but may involve the actomyosin complex. A schematic representation of how PKC may fit into the signalling pathways known to regulate cell adhesion is shown in Fig 5.30. Analysis of the recruitment of vinculin to the Triton X-100 insoluble cytoskeleton suggests that phosphorylation does not play a role in recruiting the protein to the focal adhesions. Moreover in vitro analysis of phosphorylation of vinculin by PKC isoforms further confirms that although it is a substrate for PKC in vitro, phosphorylation by PKC does not relieve the head-tail interaction and is not likely to modulate recruitment of the protein to the focal adhesion.
Figure 5.30. Schematic representation of pathways regulating cell matrix adhesion. The LPA-mediated and the fibronectin-mediated pathways are indicated. The positions of action of various inhibitors and activators described in the text are indicated in italics, agents which inhibit represented in red and agents which activate represented in green.
Chapter Six

References


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Appendix One
Acidic phospholipids inhibit the intramolecular association between the
N- and C-terminal regions of vinculin, exposing actin-binding and protein
kinase C phosphorylation sites

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Chick vinculin polypeptides expressed in Escherichia coli as glutathione S-transferase (GST) fusion proteins have been used to identify the sites involved in the intramolecular association between the 90 kDa N-terminal head and the 30 kDa C-terminal tail region of the vinculin molecule. Fusion proteins spanning vinculin residues 1-258 and 1-398, immobilized on glutathione-agarose beads, were shown to bind a C-terminal vinculin polypeptide spanning residues 881-1066 (liberated from GST by thrombin cleavage). However, the C-terminal polypeptide did not bind to a fusion protein spanning residues 399-881 or to itself. Binding was dependent on residues 167-207 within the N-terminal polypeptide, a sequence also essential for talin binding. Conversely, the 90 kDa head polypeptide was shown to bind to residues 1029-1036 in the tail region of vinculin. The association of head and tail was inhibited by acidic, but not neutral, phospholipids. Pre-incubation of vinculin with acidic phospholipids exposed the binding site for F-actin and a phosphorylation site for protein kinase C. The phosphorylation site was located in the tail region of the vinculin molecule. These results raise the possibility that acidic phospholipids play a role in regulating the activity of vinculin and therefore the assembly of both cell-cell and cell-matrix adherens-type junctions.

INTRODUCTION

Vinculin is a highly conserved 117 kDa cytoskeletal protein containing 1066 amino acids [1] found in both cell-cell and cell-extracellular matrix adherens-type junctions. In such junctions it is thought to be one of a number of interacting proteins which link the cytoplasmic face of adhesion receptors of the cadherin or integrin families to the actin cytoskeleton. Rotary shadowing electron microscopy suggests that vinculin is comprised of a globular head and an extended tail [2], although the tail is not always visible. The globular head region contains the N-terminus of the protein, a talin-binding region within residues 1-258 [3,4], and three 112-residue repeats of unknown function (Figure 1). Evidence for an a-actinin-binding site between residues 1 and 107 has recently been presented [5]. A proline-rich region, which spans residues 837-878 and contains two sites for V8-protease [6], is thought to separate the globular head from the extended tail. The C-terminal tail region has been shown to contain a binding site (residues 893-1016) for F-actin [7] and for paxillin (residues 978-1000) [8], another protein localized to focal adhesions.

Recent studies have provided compelling evidence for an intramolecular association between the tail region of vinculin and the globular head [9,10]. The tail has also been shown to bind acidic phospholipids [11]. In the present study, we have defined the regions responsible for the intramolecular association within the vinculin molecule and have shown that this association is relieved by acidic phospholipids exposing both an F-actin-binding site and a site recognized by protein kinase C (PKC).

MATERIALS AND METHODS

Rat brain PKC (1000 units/mg), isopropyl β-D-thiogalactoside and PtdIns(4)P and Ptd(4,5)P2, supplied as the diammonium salts, were obtained from Calbiochem (Nottingham, U.K.). Phosphatidylinositol was obtained from Lipid Products (S. Nutfield, Surrey, U.K.). Ampicillin, rabbit muscle actin, thrombin, PMSF, V8-protease-agarose beads, brain lipids, glutathione-agarose beads and phorbol 12-myristate 13-acetate (PMA) were from Sigma (Poole, Dorset, U.K.). [y-32P]ATP with a specific activity of 111 TBq per mmol was from Amersham International (Little Chalfont, Bucks., U.K.).

Abbreviations used: GST, glutathione S-transferase; PMA, phorbol 12-myristate 13-acetate; PKC, protein kinase C.

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Expression of vinculin polypeptides as glutathione S-transferase (GST) fusion proteins

All of the chick vinculin fusion proteins used in this study, with the exception of one spanning residues 881–1036, have been described previously [4]. This latter fusion protein was generated by PCR using a cDNA encoding vinculin residues 881–1066 as template, and primers containing a 5' BamH1 site and a 3' EcoR1 site. The PCR product was cloned into BamH1–EcoR1-cut pGEX-2T (Pharmacia). The identity of the constructs used throughout this study was confirmed by double-strand sequencing using primers 5' and 3' to the pGEX cloning site. GST-fusion proteins expressed in Escherichia coli (strain JM101) were purified from 250 ml cultures by standard methods [12]. Briefly, after induction with isopropyl β-D-thiogalactoside, cells were harvested by centrifugation, resuspended in 5 ml of PBS/1% Triton X-100, lysed by sonication and the solubilized fusion proteins purified by adsorption on glutathione-agarose beads. Beads were washed thoroughly with PBS to remove contaminating E. coli proteins, and 1 mM PMSF was added to all buffers immediately before use.

Purification of vinculin and production of vinculin polypeptides by limited proteolysis

Vinculin was purified to homogeneity from adult chicken gizzard as described by Evans et al. [13]. The 90 kDa N-terminal head fragment of vinculin was produced by incubation (3 h at 37 °C) of intact vinculin with V8-protease immobilized on agarose beads, as described by Price et al. [6]. A C-terminal vinculin tail polypeptide (residues 881–1066) was expressed as a GST-fusion protein in E. coli and the vinculin polypeptide was liberated by thrombin cleavage whilst the fusion protein was still attached to glutathione-agarose. Cleavage was carried out in 50 mM Tris/HCl, pH 7.5, containing 150 mM NaCl/2.5 mM CaCl₂ and an enzyme-to-substrate ratio of 1:500. Digestion was carried out at 37 °C for up to 2 h with continual rotation of the sample.

Binding of vinculin polypeptides to vinculin fusion proteins

Vinculin polypeptides were incubated (30 min at 37 °C) with vinculin GST-fusion proteins bound to glutathione-agarose with continuous inversion. Incubations were performed in PBS containing 0.1% (w/v) BSA to eliminate non-specific protein–protein interactions. The concentration of each fusion-protein bound to the agarose beads was estimated by SDS/PAGE and staining with Coomassie Blue, and approximately the same amount of each fusion protein was used in each assay. After incubation, the beads were pelleted in a microfuge, the supernatants removed and the pellets washed once with PBS/0.1% BSA (1.5 ml) and a further two times with PBS. Proteins bound to glutathione-agarose were dissolved by boiling in electrophoresis sample buffer and were analysed by SDS/PAGE [14].

Preparation of liposomes

Lipid stocks were dissolved in chloroform and kept at −20 °C. For liposome preparation, lipids were dried under vacuum for 30 min before sonication (2 × 5 min) in a buffer containing 20 mM Tris/HCl (pH 7.2)/0.2 mM EGTA (10 mg of lipid/ml). The clear dispersions were centrifuged at 100000 g for 20 min and the supernatant incubated with the vinculin tail polypeptide for 20 min at room temperature at a molar ratio of 2:1 (lipid:tail). Lipid–tail mixtures so prepared were used directly in the binding assay described above.

Actin co-sedimentation assay

Rabbit muscle G-actin (Sigma) was dissolved in buffer A [10 mM Tris/HCl (pH 8)/0.2 mM ATP/0.2 mM dithiothreitol/0.2 mM CaCl₂] at a protein concentration of 5 mg/ml. Assays were carried out in airfuge tubes (Beckman) and contained 5 µl of actin, 10 µl of 10 × buffer A, 5 µl of buffer B (60 mM MgCl₂/2 M NaCl) and 10–20 µg of vinculin in a final volume of 100 µl. Samples were mixed by pipetting, incubated at room temperature for 60 min, then centrifuged at 100000 g for 30 min. The resulting supernatants and pellets were analysed by SDS/PAGE. Where appropriate, intact vinculin was pre-incubated (20 min at room temperature) with lipid prepared as described above, and any aggregates removed by centrifugation at 100000 g before use in an actin sedimentation assay.

Phosphorylation of vinculin and vinculin polypeptides

Vinculin (1–2 mg/ml) was incubated with PKC (0.11 units/ml final concentration), 140 µM ATP (approx. 3000 c.p.m./nmol), 5 mM MgCl₂ and 0.1 µg/ml PMA at 37 °C. At the times indicated, aliquots were mixed with SDS/PAGE sample buffer and were analysed in SDS gels. In some cases, intact vinculin was pre-incubated (20 min at room temperature) with various lipids before use in phosphorylation assays in vitro.

RESULTS

Vinculin residues 1–258 contain a binding site for the C-terminal region of the molecule

To define the site within the 90 kDa N-terminal globular head region of vinculin that is recognized by the C-terminal tail region of the molecule, we first expressed a fusion protein spanning...
I the vinculin molecule is known to contain the tail-binding site, within the first 258 residues of the head domain. This region of the vinculin molecule is known to contain the tail-binding site, an activity which is abolished when residues 167–207 are deleted [4]. Interestingly, a fusion spanning residues 1–398, but lacking residues 167–207, was also unable to bind to the C-terminal polypeptide (Figure 2C).

Identification of the binding site for the N-terminal region of vinculin in the C-terminal tail region of the molecule

To further define the region in the C-terminal tail of vinculin responsible for the head–tail interaction, a series of C-terminal deletion mutants based on the fusion protein 881–1066 were constructed (Figure 1). The ability of these GST-fusion proteins to bind to the 90 kDa vinculin polypeptide generated by V8-protease cleavage of vinculin was assayed by co-sedimentation. The results show that fusion proteins containing residues 881–1066 and 881–1036 were both able to bind the 90 kDa vinculin head fragment (Figure 3). However, removal of a further eight amino acid residues to yield a GST-fusion protein spanning residues 881–1028 totally abolished binding. The GST-fusion proteins 881–1021, 881–1012 and 881–1000 were also unable to bind the head domain (results not shown). We conclude that the sequence between residues 1028 and 1036 is essential for binding of the C-terminal region of vinculin to the N-terminal region of the molecule.

Effect of lipids on the vinculin head–tail interaction

The C-terminal tail region of the vinculin molecule has recently been shown to contain a binding site for acidic phospholipids [11]. The effect of pre-incubating the free tail polypeptide (residues 881–1066) with various phospholipids on its ability to bind to a fusion protein spanning vinculin residues 1–258 was therefore investigated. Pre-incubation of the vinculin tail polypeptide with phosphatidylserine or brain lipids for 20 min markedly inhibited head–tail binding (Figure 4), as did PtdIns4P and Ptd(4,5)P₂ (results not shown). In contrast, the neutral lipid phosphatidylcholine (Figure 4) or phosphatidylethanolamine (results not shown) had no effect on the interaction. Quantitative analysis of the effects of various lipids on the vinculin head–tail interaction revealed that mixed brain lipids were more effective than either phosphatidylserine, PtdIns4P or Ptd(4,5)P₂ alone (Figure 4), raising the possibility that the effect is either due to a combination of acidic phospholipids or that a novel phospholipid present in brain has a greater potency than any of the individual phospholipids used in this study.

Acidic phospholipids expose the F-actin-binding site in vinculin

To establish whether binding of acidic phospholipids to the C-terminal tail region of vinculin caused any change in the activity
of the vinculin molecule, we assayed their effect on the ability of vinculin to bind to, and therefore co-sediment with, F-actin. Vinculin showed little or no ability to co-sediment with F-actin under the conditions of the assay (Figure 5A). However, pre-incubation of vinculin with acidic phospholipids such as phosphatidyserine or Ptd(4,5)P₂ led to the majority of the added vinculin co-sedimenting with F-actin. The effect was most marked with Ptd(4,5)P₂, whereas phosphatidylcholine had little or no effect. The free vinculin tail polypeptide bound to F-actin even in the absence of acidic phospholipids (results not shown), an observation consistent with the idea that the vinculin head–tail interaction normally obscures the actin-binding site.

**Effect of acidic phospholipids on the phosphorylation of vinculin**

Vinculin has been reported to be a substrate for PKC both in vitro [15] and in vivo [16]. However, our attempts to phosphorylate intact vinculin in vitro using rat brain PKC (Figure 6) or recombinant PKC-α and -δ isoforms (results not shown) were unsuccessful. Because acidic phospholipids have a marked effect on the intramolecular interactions within vinculin, we tested the effect of these lipids on vinculin phosphorylation by rat brain PKC. Pre-incubation of vinculin with a mixture of brain lipids, PtdIns4P and Ptd(4,5)P₂ resulted in readily detectable vinculin phosphorylation which increased with time. Pre-incubation of vinculin with phosphatidyserine produced a similar though somewhat lesser response, whilst phosphatidylcholine produced little or no effect. The activity of the PKC was not substantially altered by the presence of the lipids as measured by the degree of PKC autophosphorylation (results not shown). No phosphorylation was observed when vinculin was incubated in the absence of PKC, whether lipids were present or not.

**The C-terminal tail region of vinculin contains a cryptic PKC phosphorylation site**

In order to establish which regions of the vinculin molecule contain the residues phosphorylated by PKC, we repeated the in vitro phosphorylation experiments using recombinant vinculin polypeptides. Fusion proteins spanning residues 1–398 and 399–881 were not substrates for PKC, but a fusion protein containing the C-terminal tail of vinculin was heavily phosphorylated in a manner that was independent of added lipid (Figure 7). The results suggest that the PKC phosphorylation site in vinculin is normally masked by the intramolecular association of the head with the tail.

**DISCUSSION**

In the present study, we have shown that vinculin residues 1–258 and 1029–1036 are required for the intramolecular association.
Acidic phospholipids inhibit vinculin head-tail binding

between the 90 kDa N-terminal head region and the 30 kDa C-
terminus of the protein. This association is regulated by acidic phospholipids which expose a cryptic F-actin-binding site in the C-terminal tail. The experiment measuring phospholipid binding suggests that acidic phospholipids cause the major PKC phosphorylation site to become inaccessible to the PKC enzyme. This, however, is contrary to the conclusion that vinculin is not a good substrate for PKC.

The observation that the vinculin tail and talin compete for binding to the actin skeleton is more heavily phosphorylated than that in the soluble compartment. Phosphorylation is important in controlling the function of vinculin. Vinculin has been reported to be phosphorylated by PKC in vitro, and vinculin phosphorylation in both chick embryo fibroblasts and Swiss 3T3 cells has been shown to increase upon treatment with phorbol esters and calcium-elevating agents, although the levels of phosphorylation observed in these experiments were low. Our studies show that vinculin is not a good substrate for various PKC isoforms, at least in vitro, but that phosphorylation is significantly increased by acidic phospholipids. Using recombinant proteins we have shown that the major PKC phosphorylation site is within the basic tail region of the molecule. Inspection of the vinculin tail sequence reveals several serine and threonine residues in the correct context for PKC phosphorylation sites (Arg/LysXXSer/ThrXXArg/Lys), as well as potential sites for cAMP-dependent protein kinase, cGMP-dependent protein kinase, AMP-activated protein kinase, casein kinase II and proline-dependent protein kinases. Cyanogen bromide cleavage of the vinculin tail polypeptide, phosphorylated in vitro by PKC in the presence of γ32P ATP, liberated a single labelled polypeptide of 9 kDa. A polypeptide of this size can only be generated by cleavage at methionine residues 933 and 1007. This region (residues 934-1006) contains two serines (Ser-941 and Ser-999) within the focal adhesion targeting sequence (1000-1028) within the molecule.

The assembly of focal adhesions in response to agents such as lysophosphatidic acid and bombesin, as well as ligand binding to integrins (S. T. Barry, A. J. Ridley, H. M. Flinn, M. I. Humphries and D. R. Critchley, unpublished work), is dependent on rho, a small GTP-binding protein. Interestingly, rho has been shown to regulate a phosphatidylinositol 4-phosphate kinase enzyme in various cells [27]. The enzyme decapitates vinculin, which is phosphorylated by PKC in the presence of γ32P ATP, liberated a single labelled polypeptide of 9 kDa. A polypeptide of this size can only be generated by cleavage at methionine residues 933 and 1007. This region (residues 934-1006) contains two serines (Ser-941 and Ser-999) within the focal adhesion targeting sequence (1000-1028) within the molecule.

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The RhoA-dependent assembly of focal adhesions in Swiss 3T3 cells is associated with increased tyrosine phosphorylation and the recruitment of both pp125FAK and protein kinase C-δ to focal adhesions

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SUMMARY

Mouse Swiss 3T3 fibroblasts cultured in serum-free medium lose their actin stress fibres and vinculin-containing focal adhesions, a process that can be reversed by the addition of serum, lysophosphatidic acid (LPA) or bombesin, and is mediated by rhoA (A. J. Ridley and A. Hall (1992) Cell 70, 389-399). We have shown that the addition of serum to these cells induces the recruitment of the cytoskeletal proteins talin, vinculin and paxillin, and the protein kinases pp125FAK and PKC-δ, to newly formed focal adhesions, and that α-actinin is distributed along the actin stress fibres associated with these structures. The newly formed focal adhesions stained heavily with an antibody to phosphotyrosine. A similar response was elicited by 100 ng/ml LPA. The effect of serum was rapid, with focal staining for paxillin largely restricted to cell margins seen within 2 minutes of serum addition, and preceding the assembly of actin filaments. Phosphotyrosine staining differed in that it was predominantly punctate and was widely distributed throughout the cell. By 5 minutes, the paxillin and phosphotyrosine staining was concentrated at the ends of actin filaments largely at the cell margins. The structures stained ranged from circular to oval, but by 10 minutes they more closely resembled the elongated focal adhesions found in cultured fibroblasts. Within 10 minutes, the addition of serum or LPA induced a marked increase in the levels of pp125FAK and paxillin immune-precipitated by an anti-phosphotyrosine antibody. The results suggest that both pp125FAK and paxillin undergo changes in tyrosine phosphorylation upon activation of rhoA, and that these changes are associated with the assembly of focal adhesions and actin stress fibres. The observation that formation of focal adhesions can be induced by the tyrosine phosphatase inhibitor vanadyl hydroperoxide is consistent with the direct involvement of tyrosine phosphorylation in the assembly process. The localisation of PKC-δ to newly formed focal adhesions suggests that serine/threonine phosphorylation may also be important in this regard.

Key words: focal adhesion formation, tyrosine phosphorylation, vinculin, talin, paxillin, pp125FAK, protein kinase C-δ

INTRODUCTION

Since the discovery that pp60^src is localised in cell-cell and cell extracellular matrix (ECM) junctions (Rohrschneider, 1980; Hamaguchi et al., 1993) in fibroblasts transformed by Rous sarcoma virus, there has been a major effort to elucidate the role of protein tyrosine phosphorylation in the regulation of cell adhesion and cell proliferation. Initial attempts to investigate the mechanism by which pp60^src disrupts cell adhesion were frustrated by a lack of structural information on the proteins involved, but characterisation of many of the adhesive glycoproteins of the ECM and identification of the integrin family of adhesion receptors has shed significant light on the molecular basis of cell-ECM interactions (Burridge et al., 1988; Hynes, 1992). It has also become clear that the ability of integrins to act as adhesion receptors is in part governed by the interaction of the cytoplasmic domains of these transmembrane proteins with the actin cytoskeleton, a link thought to be mediated by proteins such as talin, vinculin, α-actinin, tensin and paxillin (Luna and Hutt, 1992). However, the bewildering array of proteins associated with the cytoplasmic face of cell-ECM junctions has made it difficult to establish which are essential structural components and which fulfil some other role such as the regulation of cell adhesion. Obvious candidates for this latter role include protein kinase C-α (Jaken et al., 1988; Woods and Couchman, 1992), calpain II (Beckerle et al., 1987) and pp125FAK (focal adhesion) tyrosine kinase (Schaller et al., 1992; Schaller and Parsons, 1993), which have all been shown to localise to cell-ECM junctions or focal adhesions formed by cells in culture. Interestingly, when cells are allowed to attach and spread on ECM proteins, pp125FAK, paxillin and tensin are all phosphorylated on tyrosine residues coincident with the assembly of focal adhesions (Gnan and Shalloway, 1992; Burridge et al., 1992; Bockholt and Burridge, 1993). Furthermore, the tyrosine kinase inhibitor herbimycin A partially blocks these events (Burridge et al., 1992).
1992). These results are consistent with the hypothesis that binding of ECM proteins to integrins results in the activation of an intracellular protein tyrosine kinase that is involved in the assembly of focal adhesions.

Microinjection of the small GTP-binding protein rhoA into cells has previously been shown to induce actin polymerisation (Paterson et al., 1990). Compelling evidence has also recently been presented that rhoA-sensitive focal adhesions and actin stress fibres is regulated by rhoA (Ridley and Hall, 1992), although it is unclear whether this protein is itself localised in focal adhesions. In the present study we have asked whether the rhoA-dependent assembly of focal adhesions induced by serum and LPA also involves the rhoA-dependent protein tyrosine kinase that has been ADP-ribosylated in vitro. In the present study we have asked whether the rhoA-dependent assembly of focal adhesions induced by serum and LPA also involves the rhoA-dependent protein tyrosine kinase that has been ADP-ribosylated in vitro.

**MATERIALS AND METHODS**

**Cell culture**

Mouse Swiss 3T3 fibroblasts were obtained from the European Collection of Animal Cell Cultures (Porton Down, Salisbury, SP4 0JG, UK) and cultured in Dulbecco’s Modified Eagle’s Medium (DMEM) (Gibco, Paisley, Scotland) supplemented with 10% foetal calf serum (FCS) (Advanced Protein Products, Bicester, Oxford, UK) and antibiotics/antimycotics (Gibco). For immunofluorescence studies, cells were grown to 90% confluence on 1 cm diameter glass coverslips. For experiments involving western blotting and antimycolics/antibiotics (Gibco). For immunofluorescence studies, cells were grown to 90% confluence on 1 cm diameter glass coverslips. For experiments involving western blotting the coverslips were mounted on glass slides in 90% (v/v) glycerol and examined using a Zeiss Axiophot epifluorescence microscope. Photographs were taken on Ilford HPS Plus film (ASA 400) uprated to ASA 1600 during development.

**Western blot analysis**

Cell monolayers were washed once with PBS (4°C) and whole cell lysates were prepared by scraping the contents of the dish into 200 μl of protein loading buffer (0.1 M Tris, pH 6.8, 15% glycerol, 8% SDS, 9% β-mercaptoethanol and Bromophenol Blue) containing 1 mM sodium orthovanadate. Lysates were boiled immediately (5 minutes) and stored at -20°C. The proteins present in equal volumes of cell lysates were resolved in 8% SDS-polyacrylamide gels and then electroblotted to Hybond-C Extra nitrocellulose (Amersham). Blots were stained with Ponceau S to confirm that protein loading was equal in all lanes. Filters were soaked in TBS (pH 7.5) to remove the Ponceau S, and incubated in TBS (pH 7.5) containing 5% dried skimmed milk and 0.1% Tween-20 for 1 hour to block excess protein binding sites. Filters were then incubated (1 hour) with the appropriate primary antibody diluted in the same buffer, followed by either biotinylated anti-rabbit antibodies conjugated to horseradish peroxidase (Amersham) diluted 1:5000. The proteins were detected using ECL chemiluminescence kit (Amersham) and the light emitted was detected with Fuji medical X-ray film. Where necessary, the primary and secondary antibodies were stripped from the filter as per manufacturer’s instructions, and the filters reprobed with additional antibodies.

**Immune-precipitation**

Cells from 9 cm dishes were scraped into 200 μl of RIPA buffer (0.01 M Tris, pH 7.0, 0.15 M NaCl, 2 mM EDTA, 1 mM sodium orthovanadate, 0.1% SDS, 1% NP40, 1% sodium deoxycholate and 2 mM PMSF), the DNA was sheared by passage through a fine needle and cell debris was removed by centrifugation (MSE microcentaur microfuge; 13,000 rpm for 5 minutes). To immune-precipitate vinculin and β-integrin, the lysate was preabsorbed with 30 μl Protein A or G coupled to agarose beads for 2 hours at 4°C. The beads were pelleted, washed several times with NET buffer (0.05 M Tris, pH 7.0, 150 mM NaCl, 5 mM EDTA, 0.1% BSA, 0.1% SDS, 0.1% sodium deoxycholate and 0.5% NP40) and boiled for 5 minutes in 100 μl of protein loading buffer. Equal volumes of the solubilised material were analysed in 8% SDS-polyacrylamide gels, and the proteins were blotted to nitrocellulose. Filters were stained with Ponceau S to ensure that equal levels of protein were present in all lanes. The success of the immune-precipitation was confirmed by staining the filters for either vinculin or β-integrin, and the presence of phosphorytrosine in these proteins was investigated using an anti-phosphotyrosine antibody.

Phosphotyrosine-containing proteins were precipitated with an anti-phosphotyrosine antibody conjugated to agarose beads (Sigma). A 150 μl sample of lysate was incubated with 15 μl of the beads for 3 hours at 4°C, and the beads were pelleted and washed 3x in RIPA buffer. The phosphotyrosine-containing proteins were then eluted from the beads by boiling in 80 μl protein loading buffer, and the samples were then analysed for the presence of pp125FAK and paxillin, by western blotting.

**Antibodies**

A monoclonal anti-phosphotyrosine antibody (Sigma product number P3300, clone PT-66) was used at 1:2000 for western blot analysis and at 1:1000 for immunofluorescence. Phosphotyrosine-containing proteins were immune-precipitated with the same antibody conjugated to agarose beads (Sigma). A polyclonal anti-β-integrin antibody (diluted 1:20 for immunoprecipitation) and an affinity-purified polyclonal anti-β-integrin antibody (diluted 1:2000 for western blotting) were obtained from Chemicon, Cricklewood, London, UK (product number AB-3).
Serum-induced assembly of focal adhesions in Swiss 3T3 cells

RESULTS

Serum-starved Swiss 3T3 cells assemble focal adhesions in response to addition of serum

In agreement with the previous results of Ridley and Hall (1992), removal of serum from the culture medium of Swiss 3T3 cells results in loss of actin stress fibres and vinculin-containing focal adhesions (Fig. 1A,B), a process that is dramatically reversed within 20 minutes following the re-addition of low levels (0.2%) of foetal calf serum (Fig. 1C,D). The cytoskeletal proteins talin (Fig. 1E-H) and paxillin (Fig. 2A-D) are similarly recruited to the newly formed focal adhesions, whereas α-actinin is distributed along the actin-stress fibres that emanate from these structures (Fig. 1E-L). The recently discovered focal adhesion kinase pp125FAK is also located at the ends of the newly assembled actin filaments (Fig. 2E-H), and staining cells with an antibody to phosphotyrosine showed that the newly formed focal adhesions are enriched in phosphotyrosine-containing proteins (Fig. 2I-L). Serum-starved cells frequently displayed a characteristic punctate-staining pattern for actin throughout the cytoplasm along with plasma membrane staining (for example, see Fig. 1E). In contrast, none of the focal adhesion proteins could be visualised by immunofluorescence in serum-starved cells (Fig. 1E). Whilst some of this staining was clearly associated with the ends of actin filaments, in other cases the phosphotyrosine staining seemed to correspond to the faint punctate actin staining within the body of the cell (Fig. 4E,F). After 5 minutes the phosphotyrosine staining was associated with larger focal adhesions and was restricted to the ends of actin filaments, predominantly at the cell margins (Fig. 4E,F).

There was little difference in this staining pattern after 10 minutes (Fig. 4G,H). These results show that the assembly of the focal adhesion triggered by the addition of serum is very rapid and is associated with tyrosine phosphorylation of the components of focal adhesions.

Lysophosphatidic acid-induced formation of focal adhesions

The bioactive lipid lysophosphatidic acid (LPA) has been shown to induce the formation of actin stress fibres in serum-starved Swiss 3T3 cells (Ridley and Hall, 1992). Addition of LPA (100 ng/ml) to serum-starved Swiss 3T3 cells triggered the formation of actin stress fibres and the recruitment of vinculin, paxillin and pp125FAK to filament ends within 20 minutes, and the newly formed focal adhesions were again strongly stained with an antibody to phosphotyrosine (Fig. 5).

The levels of pp125FAK and paxillin precipitated with an anti-phosphotyrosine antibody increase during the serum- and LPA-induced assembly of focal adhesions

Confluent dishes of serum-starved Swiss 3T3 cells were treated with 0.2% foetal calf serum or LPA (100 ng/ml) for 10 or 20 minutes, and equal amounts of cell lysates were analysed by SDS-PAGE and western blotting using the antibody to phosphotyrosine. Both serum and LPA induced a rapid and marked increase in tyrosine phosphorylation of a protein with an apparent molecular mass of 116 kDa, as well as increased phosphorylation of a diffusely staining band with an apparent molecular mass of 60-70 kDa (Fig. 6A). There was also increased tyrosine phosphorylation of an 80 kDa protein. Two of the phosphotyrosine-containing proteins are similar in size to proteins that have been shown to become phosphorylated on tyrosine during cell spreading on fibronectin (Burridge et al., 1992), namely the 125 kDa focal adhesion kinase (pp125FAK) and paxillin (68 kDa).

To characterise further those proteins that become tyrosine phosphorylated during the serum- and LPA-induced formation of focal adhesions, serum-starved and treated cells were lysed and phosphotyrosine-containing proteins precipitated with an antibody to phosphotyrosine conjugated to agarose beads. The precipitate was then analysed by SDS-PAGE and western blotting using the antibody to phosphotyrosine. Both serum and LPA induced a rapid and marked increase in tyrosine phosphorylation of a protein with an apparent molecular mass of 116 kDa, as well as increased phosphorylation of a diffusely staining band with an apparent molecular mass of 60-70 kDa (Fig. 6A). There was also increased tyrosine phosphorylation of an 80 kDa protein. Two of the phosphotyrosine-containing proteins are similar in size to proteins that have been shown to become phosphorylated on tyrosine during cell spreading on fibronectin (Burridge et al., 1992), namely the 125 kDa focal adhesion kinase (pp125FAK) and paxillin (68 kDa).
blotting using antibodies to pp125FAK and paxillin. The amounts of both pp125FAK (Fig. 6B) and paxillin (Fig. 6C) precipitated by the anti-phosphotyrosine antibody were markedly increased in cells treated with serum or LPA. The simplest interpretation of these results is that pp125FAK and paxillin become phosphorylated on tyrosine residues during serum and LPA-induced assembly of focal adhesions in Swiss 3T3 cells. However, the results do not formally exclude the possibility that pp125FAK and paxillin are not themselves phosphorylated on tyrosine residues, and are precipitated by the anti-phosphotyrosine antibody because they bind to other phosphotyrosine-containing proteins. To exclude this possibility, we have attempted to analyse the phosphotyrosine content of pp125FAK and paxillin directly immune-precipitated with the appropriate mouse monoclonal antibody, but in our hands the antibodies are inefficient at precipitating the mouse proteins.

Other proteins known to be associated with focal adhesions also have molecular masses in the 116 kDa range, including vinculin and the β1 subunit of integrins. The phosphorylation of these two proteins was investigated by directly immune-precipitating the proteins from cell lysates with the appropriate primary antibody and Protein A-agarose. The precipitates were then analysed by SDS-PAGE and western blotting with the anti-phosphotyrosine antibody. Neither vinculin (Fig. 7A) nor the β1 integrin subunit (Fig. 7B) appeared to be tyrosine phosphorylated under the conditions of assay. The presence of

Fig. 1. Serum-induced formation of actin filaments and focal adhesions in Swiss 3T3 cells. Localisation of vinculin, talin and α-actinin. Semi-confluent Swiss 3T3 cells were cultured in serum-free medium for 16 hours. Some of the cells were then fixed and stained for F-actin (A,E,I) and double-stained for vinculin (B), talin (F), or α-actinin (J). Other cells were treated with fresh medium containing 0.2% foetal calf serum for 20 minutes, and fixed and stained for F-actin (C,G,K) with double staining for vinculin (D), talin (H), or α-actinin (L). Bar, 5 μm.
Serum-induced assembly of focal adhesions in Swiss 3T3 cells

Vinculin and the β1 integrin subunit on the blot was confirmed by reprobing the filters with antibodies to each of the two proteins in turn. Immune-precipitation with the β1 integrin antibody should co-precipitate associated α-subunits, although we have not confirmed this directly by western blotting. Assuming that this is the case, we have found no evidence for tyrosine phosphorylation of these α-subunits.

**Vanadyl hydroperoxide induces assembly of actin stress fibres and focal adhesions in serum-starved cells**

If the tyrosine phosphorylation of proteins is a causal factor in the assembly of actin stress fibres and focal adhesions, one might expect tyrosine phosphatase inhibitors to trigger this process. As predicted, serum-starved Swiss 3T3 cells treated with 50 μM vanadyl hydroperoxide for 20 minutes contained numerous actin stress fibres and vinculin-containing focal adhesion (Fig. 8C,D) compared with untreated cells (Fig. 8A,B). Interestingly, the stress fibres formed were thicker and more numerous than those found in cells treated with serum or LPA. Similarly, the number of focal adhesions formed in response to vanadyl hydroperoxide treatment was greater than induced by serum or LPA.

**PKC-δ is recruited to newly formed focal adhesions**

Protein kinase C (PKC-δ) has previously been implicated in...
Fig. 3. Time course of the formation of actin filaments and paxillin-containing focal adhesions. Serum-starved Swiss 3T3 cells were treated with 0.2% FCS for 0 minutes (A,B), 2 minutes (C,D), 5 minutes (E,F) or 10 minutes (G,H) and the cells fixed and double-stained for F-actin (A,C,E,G) or paxillin (B,D,F,H). Bar, 5 μm.
Fig. 4. Time course of the formation of actin filaments and phosphotyrosine-containing focal adhesions. Serum-starved Swiss 3T3 cells were treated with 0.2% FCS for 0 minutes (A,B), 2 minutes (C,D), 5 minutes (E,F) or 10 minutes (G,H) and the cells fixed and double-stained for F-actin (A,C,E,G) or phosphotyrosine (B,D,F,H). Bar, 5 μm.
Fig. 5. Lysophosphatidic acid-induced formation of actin filaments and focal adhesions in Swiss 3T3 cells. Swiss 3T3 cells cultured in serum-free medium for 16 hours were fixed and stained for F-actin (A,E,I,M) and double-stained for vinculin (B), paxillin (F), pp125FAK (J) or phosphotyrosine (N). Other cells were treated with fresh medium containing 100 ng/ml lysophosphatidic acid for 20 minutes and fixed and stained for F-actin (C,G,K,O) with double staining for vinculin (D), paxillin (H), pp125FAK (L) or phosphotyrosine (P). Bar, 5 μm.
the assembly of focal adhesions during cell spreading on fibronectin (Woods and Couchman, 1992; Vuori and Ruoslahti, 1993), and PKC-α has been localised to focal adhesions in rat embryo fibroblasts (Jaken et al., 1989). However, we have not found PKC-α in the focal adhesions of Swiss 3T3 cells as judged by immunofluorescence staining, although it is clearly expressed in these cells (data not shown). Olivier and Parker (1994) have recently shown that bombesin, PDGF and diacylglycerol treatment of Swiss 3T3 cells results in the redistribution of PKC-δ to the Triton X-100 cytoskeleton, and we therefore studied the subcellular distribution of this isoform during the assembly of focal adhesions in Swiss 3T3 cells. In serum-starved Swiss 3T3 cells lacking focal adhesions and actin stress fibres, PKC-δ immunostaining was largely restricted to the perinuclear region, with weak staining at points of cell-cell contact (Fig. 9A,B). Interestingly, within 20 minutes of the addition of serum, PKC-δ was localised at the ends of actin stress fibres in focal adhesions, with some staining along actin filaments (Fig. 9C,D). Identical results were obtained when serum-starved cells were treated with LPA (data not shown).

**DISCUSSION**

Culturing Swiss 3T3 cells overnight in serum-free medium results in the loss of actin stress fibres and vinculin-containing focal adhesions (Ridley and Hall, 1992), a process that can be reversed by addition of non-mitogenic levels of FCS (the active component of which is LPA) and the neuropeptide bombesin. Furthermore, Ridley and Hall (1992) have demonstrated that the effects of these agents are mediated via the small GTP-binding protein rhoA, and microinjection of rhoA itself triggers the reappearance of actin stress fibres and focal adhesions. This system therefore has great potential for studying the regulation of cell adhesion, although we have yet to find a cell line other than Swiss 3T3 cells that responds to serum starvation in quite the same way.
We have now shown that addition of 0.2% FCS to serum-starved Swiss 3T3 cells induces recruitment of the cytoskeletal proteins vinculin, talin and paxillin, as well as pp125FAK and PKC-δ, to newly formed focal adhesions, and that α-actinin is distributed along the actin filaments that are associated with these adhesions. Similar results were obtained following LPA stimulation. Unfortunately, we have been unable to analyse the effects of FCS and LPA on the distribution of β1 integrins due to the lack of an antibody suitable for immunofluorescence studies in mouse cells. We have also shown that both FCS and LPA induced a marked increase in tyrosine phosphorylation of the components of the focal adhesion as detected by immunofluorescence. The effect of FCS was rapid, with focal staining for paxillin and phosphotyrosine evident within 2 minutes of addition, preceding the assembly of clearly discernable actin stress fibres. However, the staining for paxillin and phosphotyrosine were significantly different. Paxillin staining was most prominent at the cell margins, coincident with sites that stained strongly for membrane-associated actin. In contrast, numerous small phosphotyrosine-containing structures were distributed throughout the body of the cell coincident with faint staining for actin. Larger phosphotyrosine-containing structures associated with the ends of actin stress fibres or membrane-associated actin and more closely resembling focal adhesions appeared at the cell margins. It is possible that the punctate phosphotyrosine staining represents the earliest stages in assembly of the focal adhesion, and that maturation of this structure is associated with the rapid recruitment of paxillin and other cytoskeletal proteins. We have tried to establish whether pp125FAK is associated with the punctate phosphotyrosine staining, but this has not proved possible due to the poor cross-reactivity of the pp125FAK monoclonal antibody with the mouse protein.

We have also shown that proteins with apparent molecular masses of 116 kDa, 80 kDa and 60-70 kDa undergo a marked increased tyrosine phosphorylation within 10 minutes of the addition of serum or LPA to serum-starved Swiss 3T3 cells. We have tentatively identified two of these proteins as the 125 kDa focal adhesion kinase pp125FAK and paxillin (68 kDa), which runs as a diffuse band in SDS-PAGE. It remains possible, and even likely, that other proteins that co-migrate with pp125FAK and paxillin in SDS-gels are also tyrosine phosphorylated during focal adhesion assembly. However, we have shown that vinculin (117 kDa) and the β1 integrin subunit (116 kDa) plus associated α-subunits are not phosphorylated on tyrosine during this same period. It is tempting to speculate that the phosphorylation of pp125FAK and paxillin is causally linked with the assembly of focal adhesions and stress fibres, although a more detailed analysis of the kinetics of the two events is required. However, the idea that protein tyrosine phosphorylation is directly involved in focal adhesion assembly and the formation of actin stress fibres is supported by the finding that assembly can be induced by the tyrosine phosphatase inhibitor vanadyl hydroperoxide.

The above observations show a striking parallel with those of Burridge et al. (1992), who showed that pp125FAK and paxillin were two of the major proteins undergoing tyrosine phosphorylation during cell spreading and the assembly of focal adhesions on ECM proteins. More recently, Bockholt and Burridge (1993) have shown that tensin (210 kDa) is also phosphorylated during cell spreading whereas vinculin and talin were not phosphorylated at least on tyrosine residues. However, the serum induction of focal adhesion assembly in Swiss 3T3 cells is more rapid than that observed during cell spreading on ECM proteins, making the system more suitable...
for future detailed analysis of the kinetics and mechanisms underlying the process.

Given that the serum- and LPA-induced assembly of focal adhesions in Swiss 3T3 cells is mediated via rhoA (Ridley and Hall, 1992), it is reasonable to speculate that the tyrosine phosphorylation of both pp125FAK and paxillin induced by these agents is mediated via the same mechanism. Bombesin, which also acts via rhoA to stimulate assembly of focal adhesions and actin stress fibres (Ridley and Hall, 1992), similarly induces the tyrosine phosphorylation of pp125FAK (Sinnett-Smith et al., 1993). The possibility that rhoA directly or indirectly controls the activity of pp125FAK awaits further experimentation. Interestingly, the small GTP-binding protein p21Ha-ras has recently been shown to activate the serine/threonine kinase raf-1 through a direct physical association (Vojtek et al., 1993). Whether the phosphorylation of pp125FAK is an effect of the activation of the FAK kinase itself, resulting in autophosphorylation or whether an additional kinase is involved is unclear. In platelets, binding of fibrinogen to the integrin αIIbβ3 initiates tyrosine phosphorylation of a number of proteins with apparent molecular masses of 50-68 kDa and 140 kDa (Huang et al., 1993). Phosphorylation of these proteins precedes the tyrosine phosphorylation of pp125FAK, which is dependent on platelet aggregation. These results suggest the involvement of an additional tyrosine kinase, which might in turn regulate pp125FAK activity.

Quite how the tyrosine phosphorylation of pp125FAK might regulate the assembly of focal adhesions is open to speculation. pp125FAK has been reported to be activated by phosphorylation (Guan and Shalloway, 1992) and is capable of phosphorylating paxillin at least in vitro (Turner et al., 1990) and possibly tensin (Schaller and Parsons, 1993). The fact that tensin has an SH2 domain and is reportedly able to bind vinculin and F-actin (Davis et al., 1991; Lo and Chen, 1994) suggests that it could play a central role in the assembly of the interacting network of proteins that are thought to link integrins to the actin cytoskeleton. The only known activity of paxillin is to bind to vinculin (Turner et al., 1990; Wood et al., 1994), and the affinity of this interaction might be controlled by the tyrosine phosphorylation of paxillin. Vinculin can in turn bind talin (Burridge and Mangeat, 1984; Gilmore et al., 1992; Gilmore et al., 1993) and α-actinin (Belkin and Kotelsiansky, 1987; McGregor et al., 1994), two proteins implicated in binding to the cytoplasmic domain of β1 integrins (Horwitz et al., 1986; Otey et al., 1990). The fact that we have now shown that pKC-δ is localised to newly formed focal adhesions lends support to previous suggestions that PKC, and therefore serine/threonine phosphorylation, might also be important in focal adhesion assembly and cell spreading (Jaken et al., 1989; Woods and Couchman, 1992; Vuori and Ruoslahti 1993). Both vinculin (Werth and Pastan, 1984) and talin (Litchfield and Ball, 1986; Turner et al., 1989) have previously been reported to be phosphorylated in response to PKC activation, although the effects of such phosphorylation events on the activities of these two proteins is not known. Interestingly, bombesin, PDGF and diacylglycerol have recently been shown to promote recruitment of PKC-δ to the Triton X-100 cytoskeleton of Swiss 3T3 cells (Olivier and Parker, 1994) and this PKC isoform (75-82 kDa) becomes tyrosine phosphorylated in NIH-3T3 cells treated with phorbol esters (Li et al., 1994). Furthermore, the activity of PKC-δ has been shown to increase following in vitro phosphorylation by the insulin receptor, β-PDGFR receptor and Lyn tyrosine kinases. It will be interesting to establish whether the 80 kDa protein that we have found to exhibit an increased phosphotyrosine content during the assembly of focal adhesions is indeed PKC-δ.
Whilst recent emphasis has been on the role of tyrosine phosphorylation in the assembly of focal adhesions, the tyrosine phosphorylation of focal adhesion proteins mediated by pp60C-Src has long been associated with destabilisation of these structures (Robson, 1980; David-Pfeuty and Singer, 1989; Kelle et al., 1986). Interestingly, two of the proteins that are heavily phosphorylated on tyrosine residues in Rous sarcoma virus-transformed cells include pp125FAK and pp130Gluan and Shalloway, 1992) and ppl23FAK has been shown to associate with pp60C-Src in Rous sarcoma virus-transformed cells (Cobb et al., 1994). In some transformed cells, the focal adhesion proteins form large aggregates or rosettes on the ventral surface of the cell (David-Pfeuty and Singer, 1980) and the proteins in these rosettes are heavily tyrosine phosphorylated (Glenney and Zokas, 1989). Whilst these rosettes contain vinculin and actin they are not associated with actin stress fibres. In this respect, they resemble the newly formed focal adhesions observed in this study, pp60C-Src may inhibit the decoration of newly formed adhesions with F-actin, resulting in reduced stability of the structure and reduced cell adhesion to the ECM.

It is now well documented that ligand binding to integrins can trigger a variety of intracellular events (outside-in signalling) whilst the conformation and affinity of integrins for their ligands can be regulated by intracellular mechanisms (inside-out signalling) (reviewed by Hynes, 1992; Juliano and Belkin, A. M. and Koteliansky, V. E. (1987).)

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REFERENCES


Serum-induced assembly of focal adhesions in Swiss 3T3 cells


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Introduction

Cell adhesion to the extracellular matrix (ECM) is frequently mediated by members of the integrin family of transmembrane heterodimeric glycoproteins [1]. They are linked to the actin cytoskeleton via a number of interacting proteins localized within specialized structures observed in cultured cells variously known as focal adhesions, adhesion plaques or focal contacts. Analogous structures are found in vivo including dense plaques in smooth muscle cells and the myotendinous junction in skeletal muscle [2]. Many of the interactions between proteins found in focal adhesions have been characterized using biochemical binding assays in vitro [3]. Figure 1 illustrates the potential pathways that link integrins to the actin cytoskeleton, although it remains to be established whether the interactions shown occur in vivo. Here we review recent experiments that shed light on the structure and function of the cytoskeletal proteins talin, vinculin and α-actinin, which are thought to link integrins to the actin cytoskeleton. In addition, we summarize current data on the signalling pathways implicated in the control of cell adhesion.

Talin

Talin is a cytoskeletal protein containing 2541 amino acids with a predicted molecular mass of 270 kDa [4] but an apparent molecular mass of 220 kDa when estimated by gel electrophoresis. The human talin gene has been mapped to the short arm of chromosome 9 [4a]. The domain structure of the protein is shown in Figure 2. The calcium-dependent protease calpain II, which is localized in focal adhesions in rat embryo fibroblasts [5], cleaves talin between residues 433 and 434 [4], generating a 47 kDa N-terminal head and a 190 kDa C-terminal tail. Calpain-mediated cleavage of talin occurs on thrombin activation of platelets [6], although the functional significance is unclear. The N-terminal polypeptide contains residues required for localization of talin to cell-matrix, rather than cell-cell, junctions [7], and also binds to charged lipids suggesting that it might contain a membrane interaction site [8].

On activation of normal platelets, talin translocates from the cytoplasm to a position juxtaposed to the membrane [9]. The continued ability of talin to translocate to the membrane in platelets lacking integrin GPIb-IIIa [10] is consistent with the idea that talin can interact with membranes in an integrin-independent manner. A region within the N-terminal domain of talin (residues 165-373) shows 23% sequence identity to ezrin, a member of the ezrin/radixin/moesin (ERM) family of cytoskeletal proteins [11], all of which have a putative N-terminal membrane association site. The ERM superfamily includes the NF2 tumour suppressor gene product Schwannomin/MERLIN [11]. It will be interesting to see whether talin has tumour suppressor activity. The Wistar–Furth strain of rat exhibits a high degree of tumour incidence and platelet abnormalities. This strain carries a mutation at codon 1176 (Pro→Thr) within the talin gene [12]; however, a functional correlation between this mutation and talin dysfunction is yet to be demonstrated.

A variety of binding studies in vitro demonstrate that the 190 kDa fragment of talin contains binding sites for β1-integrin [13], actin [14] and vinculin [15] (Figure 2). Only the vinculin-binding sites have been defined further, with two or possibly three vinculin-binding sites contained within residues 498-656, 852-950 and 1554-2268. These sites overlap sequences required for targeting talin to focal adhesions [16]. C-terminal residues 2337–2541 show homology with a yeast actin-binding protein Slu2P [17], and are therefore predicted to contain an actin-binding site. Talin has also been shown to have actin nucleating activity, promoting F-actin filament formation from monomeric G-actin [18], and also enhances the rate of actin cross-linking by α-actinin [19]. The fact that talin is recruited to newly forming focal adhesions at an early stage [2] suggests that it might play a key role in the assembly of actin filaments. Evidence that talin is essential to cell spreading and migration of fibroblasts on fibronectin has come from micro-injection experiments with a polyclonal antibody [20]. More recently we have shown that micro-injection of monoclonal antibodies that recognize epitopes in...
Model of a focal adhesion showing the possible protein-protein interactions identified by binding studies in vitro

Linking of the integrin receptors to the actin cytoskeleton may be achieved via the interactions of: (A) talin, vinculin and α-actinin; (B) α-actinin and vinculin or α-actinin alone; (C) talin alone. Box contains regulatory proteins.

Vinculin

Vinculin is a 116 kDa cytoskeletal protein containing 1066 amino acids, encoded by a single-copy gene on human chromosome 10q11.2-qter [27]. The complete sequences of the human, chick and nematode proteins, and a partial mouse sequence, have been published, and the organization of the human gene has been determined. The origin of the muscle-specific isoform of vinculin (metavinculin) has been attributed to alternative splicing, resulting in the insertion of an additional 68 amino acid residues between residues 915 and 916 [28]. In the electron microscope, vinculin appears to contain a globular head and an extended tail. The head contains the N-terminus of the protein, an α-actinin binding site (residues 1-107) [29], a talin-binding site (residues 1-258) [30], and three 112-residue repeats of unknown function. The head can be liberated as a 90kDa fragment from the tail (30kDa) by V8-protease cleavage within a proline-rich region (Figure 2). The 30kDa tail contains a paxillin-binding site (residues 978-1000) [31] and an F-actin binding site (residues 893-1016) [32]. Vinculin also binds to the actin-capping protein tensin [33], but the binding site has not been defined. The direct association of vinculin with α-
Protein Targeting Interactions in Signalling Processes

Figure 2

Schematic diagram showing the domain structures of talin, (a), vinculin (b) and α-actinin (c)

Proteins are drawn approximately to scale and all functional or structural regions mapped so far are indicated for each molecule.

actinin, talin, paxillin, tensin and F-actin suggests that multiple pathways exist for the linkage of integrins to the actin cytoskeleton (Figure 1).

An intramolecular association between the N-terminal head and C-terminal tail domains of vinculin has recently been demonstrated [29,32]. The head-binding site in the vinculin tail has been mapped to between residues 1029 and 1036 by using deletion mutants (J. Weekes, S. T. Barry and D. R. Critchley, unpublished work), and the tail-binding site in the head to within residues 1–258.

Interestingly, this interaction has been shown to modulate talin [34], α-actinin [29] and F-actin [32] binding to vinculin. How the head–tail interaction is regulated, and what role this plays in the regulation of cell adhesion remain to be established. Much of the total cellular vinculin is in a soluble cytoplasmic pool, and it is possible that this form is inactive owing to the head–tail association. Although phosphorylation would seem to be an obvious mechanism to regulate vinculin activity, the results on vinculin phosphorylation are confusing. It is
known that vinculin is phosphorylated on tyrosine residues in cells transformed with RSV [35] and in a calcium-dependent manner in stimulated platelets [36]. It has also been shown to be a substrate for PKC [37], and PKC-α has been localized to focal adhesions in rat embryo fibroblasts [38]. However, the stoichiometry of vinculin phosphorylation is low, although evidence has been presented that the vinculin associated with Triton X-100-insoluble material is more heavily phosphorylated. In addition, vinculin contains a C-terminal binding site for acidic phospholipids which may be important in regulating the head–tail interaction in a manner analogous to that which regulates the interaction between the catalytic domain of PKC and the pseudo-substrate-binding site. Vinculin, α-actinin and phospholipids have been shown to form a ternary complex [39]. Interestingly, evidence suggests that a degree of intramolecular interaction also exists within talin [40].

Several recent experiments have demonstrated the functional importance of vinculin in cell adhesion. Microinjection of an anti-vinculin monoclonal antibody into cultured fibroblasts results in the disruption of actin stress fibres and focal adhesions [41]. Increasing or decreasing the level of vinculin in Balb/c 3T3 fibroblasts by using overexpression [42] and anti-sense techniques [43] respectively was found to reduce or enhance adhesion and motility. We have obtained similar results in Balb/c 3T3 cells with anti-sense vinculin RNA under the control of an inducible promoter (S. J. Bolton. S. T. Barry and D. R. Critchley, unpublished work). In accordance with these findings, a mutant mouse F9 embryonal carcinoma cell line deficient in vinculin was found to have a rounded morphology and was defective in both cell–cell and cell–matrix adhesion. An adhesive phenotype was restored by the introduction of an expression plasmid encoding a full-length chick vinculin [44]. Interestingly, expression of vinculin in an SV40-transformed Balb/c 3T3 tumour cell line containing one quarter of the vinculin found in untransformed cells resulted in a significant reduction in tumorigenicity [45]. This suggests a potential role for vinculin as a tumour suppressor protein.

α-Actinin

The rod-shaped protein α-actinin forms an antiparallel homodimer with a subunit molecular mass of approx. 100kDa. The domain structure of the protein is shown in Figure 2. It is an F-actin cross-linking and bundling protein found at sites where actin is linked to a variety of intracellular structures, including focal adhesions [46]. At least three α-actinin genes have been identified, one of which encodes the smooth muscle isoform plus a non-muscle isoform that arises via alternative splicing of an exon encoding part of the first EF-hand calcium-binding motif. This may account for the different calcium sensitivities of the two isoforms with regard to actin-binding [47]. The other two genes encode skeletal muscle isoforms. Alternative splicing of the chick skeletal-muscle α-actinin gene has also been observed [48].

We have studied the ability of α-actinin to bind F-actin via its N-terminal actin-binding domain (residues 1–266), and have localized an actin-binding site to residues 120–134 within the chick smooth muscle protein [49,50]. The homologous N-terminal domains within dystrophin [49] and utrophin [51] are also capable of binding F-actin. The N-terminal region of α-actinin has also been shown to bind zyxin, an 82kDa protein also found in focal adhesions [52]. It is thought that α-actinin dimerization is mediated by the central region of the molecule, which consists of four α-helical spectrin-like repeats. The boundaries between these repeat regions have recently been revised on the basis of limited proteolysis studies with bacterially expressed proteins [53]. The use of chemical cross-linking and analytical equilibrium sedimentation has revealed that repeats 1 and 4 are important for dimerization (G. Flood, E. Kahana, A. P. Gilmore, W. B. Glatzer, A. J. Rowe and D. R. Critchley, unpublished work). The vinculin-binding site on α-actinin has been mapped to the end of the repeat domain between residues 713 and 749 [54]. β1 integrin also binds α-actinin within the repeat region [55], and an association between ICAM-1 and α-actinin has also been observed [56]. The EF-hand calcium-binding domains in Dictyostelium discoideum α-actinin have been analysed using biochemical studies in vitro and rescue studies in vivo demonstrating that the first EF-hand regulates actin binding [57].

The level of α-actinin in Balb/c 3T3 fibroblast cells has been both increased by 40–60% and reduced to 25–60% of normal by using overexpression and anti-sense techniques respectively [58]. Overexpression reduced and underexpression increased cell motility, indicating the importance of α-actinin to adhesion. Interestingly, overexpression of α-actinin reduced the tumorigenicity of SV40-transformed cells, again suggesting a role for cytoskeletal proteins as candidate tumour suppressors. Homologous recombination in D. discoideum has also been used to analyse the functional importance
of α-actinin in vivo [59]. Deletion of α-actinin alone did not result in a change in phenotype, but ablation of a second related actin cross-linking protein, ABP-120 [59], produced slime moulds that were normal apart from being unable to form fruiting bodies. This illustrates the functional redundancy apparent within the actin cross-linking family of cytoskeletal proteins. In addition, several null α-actinin *Drosophila* mutants have been identified that possess only minor muscle alterations [60].

**Cell adhesion and signal transduction**

It is now evident that several converging signalling pathways are associated with the regulation of cell adhesion (Figure 3). When fibroblasts spread on fibronectin, the activation of integrins results in the elevation of tyrosine phosphorylation on a number of different proteins, including the focal adhesion tyrosine kinase (pp125FAK), paxillin and tensin [61,62,63]. Inhibitor studies show that the spreading of fibroblasts on fibronectin and the development of focal adhesions and actin stress fibres is dependent on tyrosine kinase activity [61]. The second pathway known to regulate the assembly of focal adhesions and actin stress fibres involves the small GTP-binding protein rhoA [64]. RhoA, which can be activated by both lysophosphatidic acid (LPA) and bombesin via receptor-coupled heterotrimeric G-proteins, regulates adhesion through the action of downstream tyrosine kinases that in turn stimulate the phosphorylation of pp125FAK and paxillin [65,66]. It has been shown recently that rhoA itself is regulated by an upstream tyrosine kinase [67].

Although the proteins that exhibit changes in tyrosine phosphorylation during the adhesive response have been extensively studied, rather less is known about the role of serine/threonine phosphorylation in the regulation of cell adhesion. However, it is now clear that pp125FAK and paxillin show a marked increase in serine/threonine phosphorylation as well as tyrosine phosphorylation during cell spreading [68,69]. PKC has been impli-
cated as a potential regulator of adhesion, as inhibition of PKC function prevents cell spreading on fibronectin of both rat embryo fibroblasts [38] and Chinese hamster ovary cells [70]. Activation of PKC with phorbol 12-myristate 13-acetate promotes spreading in both cell types. The activation of PKC was seen to precede spreading and also enhanced the integrin-mediated tyrosine phosphorylation of pp125FAK and paxillin, implicating PKC as an important factor in the stimulation of tyrosine kinase activity by adhesion [70].

The induction of tyrosine phosphorylation through both integrins and rhoA is inhibited by cytochalasin D, a reagent that disrupts the actin cytoskeleton. Furthermore, Balb/c 3T3 cells, which do not possess focal adhesions or actin stress fibres as a result of vinculin down-regulation, do not show tyrosine phosphorylation of pp125FAK and paxillin upon spreading on fibronectin (S. J. Bolton, S. T. Harry and D. R. Critchley, unpublished work). This implies that focal adhesions and the actin cytoskeleton are important, if not crucial, to the signalling process via integrins. These adhesion-defective cells also exhibit much slower growth rates than normal fibroblasts. The demonstration that clustering of integrins stimulates the mitogen-activated protein kinase pathway [71] offers an explanation for this observation [63]. However, the ability of microinjected rhoA to trigger the assembly of focal adhesions containing vinculin and phosphotyrosine-containing proteins in serum-starved Swiss 3T3 cells is not compromised by cytochalasin D [71a]. Upstream LPA receptor-mediated activation of rhoA is therefore cytoskeleton dependent, whereas signalling downstream of rhoA resulting in the formation of focal adhesions can be cytoskeleton independent.

Lipid second messengers have also been implicated as regulators of processes associated with adhesion. Phosphatidylinositol 4,5-bisphosphate (PIP2) plays a role in modulating the function of actin-binding proteins such as profilin and α-actinin [72,73]. Moreover, vinculin and α-actinin isolated from Balb/c 3T3 cell lysates by immunoprecipitation have been shown to contain bound PIP2. The interaction of α-actinin with PIP2 increases the actin cross-linking capabilities of the protein [73]. The amount of PIP2 bound to α-actinin and vinculin was found to decrease on stimulation of cells with PDGF, possibly as a result of phospholipase C activation [73]. Treatment of serum-starved Swiss 3T3 cells with PDGF inhibits the LPA-induced stress fibre formation [64], perhaps as a result of the initial decrease in PIP2 levels associated with PDGF-receptor activation. This supports the view that PIP2 and other phospholipids may be involved in regulation of the actin cytoskeleton, possibly by regulating the function of actin-binding proteins. In addition, phosphatidylinositol 5'-kinase, the enzyme that generates PIP2 from PIP, has been shown to be regulated by rhoA [74]. Generation of PIP2 releases actin sequestered by profilin and the barbed end capping protein gelsolin, making actin available for polymerization, providing a possible mechanism through which rhoA could control actin polymerization.

α-Actinin also binds the p85 subunit of phosphatidylinositol 3-kinase (PI3 kinase) in a PIP2-concentration-dependent manner [75]. PI3 kinase is important in growth-factor-mediated signal transduction catalysing the formation of other novel lipid second messengers. α-Actinin may therefore be providing a platform facilitating the enzyme-substrate interaction. Interestingly, PI3 kinase has been shown to play a role in the activation of the rhoA-related GTP-binding protein rac, as a result of PDGF stimulation of serum-starved Swiss 3T3 fibroblasts [67]. The binding of the putative regulatory protein paxillin to vinculin [31] may be a further example of structural proteins providing the necessary environment for the interaction of regulatory components and generation of subsequent signalling events.

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